The Role of DiaA and SeqA Homologues in the Deep-Sea Adapted Growth of *Photobacterium profundum* SS9

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Declaration

I hereby declare that all the work presented in this thesis is my own except where otherwise stated.

Ziad W. El-Hajj

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This thesis is dedicated to my loving family for their unconditional support and for being the best family one could hope for.

Abbreviations

::	Transposon or cassette insertion
°C	Degree Celsius
AAA+	ATPases associated with various cellular activities
Amp	Ampicillin
ADP	Adenosine bis-phosphate
APS	Ammonium per-sulphate
ATP	Adenosine tri-phosphate
bp	Base pairs
BSA	Bovine serum albumin
Chl	Chloramphenicol
cAMP	Cyclic adenosine mono-phosphate
Cap	Cold acclimation protein
chr	Chromosome
CPS	Capsular polysaccharide
Cs	Cold-sensitive
Csp	Cold shock protein
Dam	DNA adenine methylase
DAPI	4',6-Diamidino-2-phenylindol
dH_2O	Distilled water
DiaA	DnaA initiator-associating factor
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOC	Sodium deoxycholate
EcDiaA	*E. coli* DiaA
EcSeqA	*E. coli* SeqA
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharide
FACS	Fluorescent-activated cell sorting
FITC	Fluorescein isothiocyanate
Fts	Filamentous temperature sensitive
g	Gram

g	Relative centrifugal force
Gmh	*Glycero-manno*-heptose
GPa	Gigapascal
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropylthiogalactopyranoside
Kan	Kanamycin
KAS	β-ketoacyl-acyl carrier protein synthase
kb	Kilobase pairs
LB	Luria-Bertani
LGT	Low gelling temperature
LPS	Lipopolysaccharide
Mdh	Malate dehydrogenase
MPa	Megapascal
mRNA	Messenger ribonucleic acid
MUFA	Mono-unsaturated fatty acid
OD_{600}	Optical density at 600nm
Omp	Outer membrane protein
ORF	Open reading frame
ori	Origin of replication
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PpDiaA	*P. profundum* DiaA
PpSeqA	*P. profundum* SeqA
PUFA	Poly-unsaturated fatty acid
RBS	Ribosome binding site
RIDA	Regulation inactivation of DnaA
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
Rif	Rifampicin
RNA	Ribonucleic acid
rpo	RNA polymerase sigma factor

SDS	Sodium dodecyl sulfate
Strep	Streptomycin
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
Tn	Transposon
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Ts	Temperature-sensitive
UV	Ultraviolet
WT	Wild-type

Abstract

The mechanism of high pressure-adapted growth in the deep-sea bacterium *Photobacterium profundum* SS9 is poorly understood. To gain further insights, two *P. profundum* SS9R mutants were investigated. FL23 (*pbpra3229*::m-Tn*10*) and FL28 (*pbpra1039*::m-Tn*10*) had been previously characterised as high pressure-sensitive and pressure-enhanced, respectively. FL23 had a growth defect at atmospheric pressure but failed to show high pressure-adapted growth on solid agar. Pbpra3229 is 75 % identical to *E. coli* DiaA (stimulator of DNA replication and critical for the timely initiation of replication) and 45% identical to *E. coli* GmhA (essential for lipopolysaccharide core biosynthesis), which led to an investigation into whether either process was affected in FL23. However, the lipopolysaccharide of FL23 and its parent strain were identical, which suggests that Pbpra3229 is not a GmhA homologue. In contrast, the *pbpra3229* and *E. coli diaA* genes were functionally interchangeable and both restored the timing of DNA replication in an *E. coli diaA* mutant. FL28 had growth and morphological defects at high pressure, but both phenotypes were exacerbated at atmospheric pressure. Pbpra1039 is 55% identical to *E. coli* SeqA, which is a negative regulator of DNA replication and also essential for timely initiation. Pbpra1039 was shown to be a functional homologue of *E. coli* SeqA, as *pbpra1039* partially complemented the DNA replication defect of an *E. coli seqA* mutant. Combined, these findings provide evidence that Pbpra3229 is a DiaA homologue, whereas Pbpra1039 is a cold adapted SeqA homologue, and that both positive and negative regulation of initiation of DNA replication are essential for the ability of *P. profundum* SS9 to adapt to deep-sea conditions. A marine metagenomic library was also screened for clones that produced novel cell envelope polysaccharides and tools were developed to identify cell envelope polysaccharides in *P. profundum* SS9.

Chapter 1

Introduction

1.1. The Deep Sea

Although the deep-sea comprises the largest portion of the Earth's biosphere by volume and is thought to contain over half of all marine prokaryotic cells (Whitman *et al*, 1998), our knowledge of the microbial diversity within this environment is rather poor. While terrestrial and surface marine environments are both characterised by a relatively constant pressure (0.1MPa at sea level, and 0.06MPa at the top of Mount Everest) and fluctuating temperatures, the deep sea dramatically differs in this respect, with an average constant temperature of 3°C (Bruun, 1957) and a pressure that increases by 0.1MPa for every 10m depth (Saunders and Fofonoff, 1976). High pressure is one of the most distinctive features of the deep sea, since it can reach values as high as 110MPa in the Mariana Trench (Figure 1-1), the deepest point of the Earth's oceans (11km deep). Even at the depth of the Mariana Trench, extremophiles are able to survive and grow (Yayanos *et al*, 1981). Microorganisms that have adapted to grow optimally in the deep sea are usually both piezophiles (pressure-adapted; formerly known as barophiles) and psychrophiles (cold-adapted). A number of the identified piezophiles belong to the γ-proteobacteria, and are closely related to *Shewanella* or *Photobacterium* species found in surface water (DeLong *et al*, 1997).

1.1.1. Cold temperature and psychrophiles

Like most other mesophiles, *Escherichia coli* grows optimally at 37°C and is

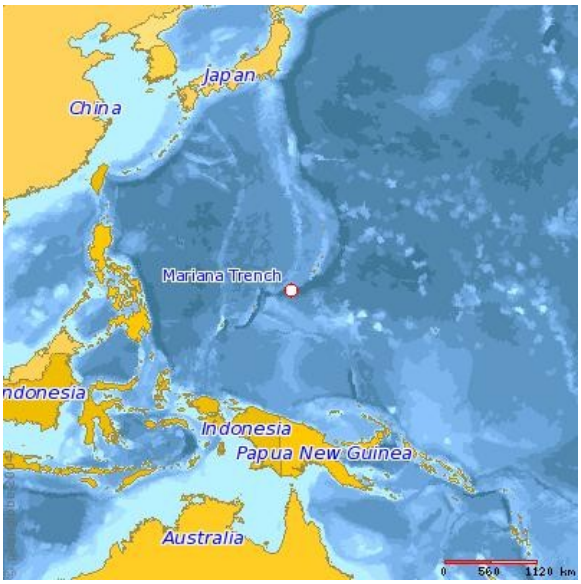


Figure 1-1. The Mariana Trench. Geographic location of the Mariana Trench (11°21' N and 142°12' E). Challanger Deep, the bottom of the Trench, is the deepest point of the ocean. (source: Wikipedia)

unable to grow at all at temperatures below 8°C (Shaw *et al*, 1971). By contrast, psychrophiles are capable of growing at temperatures around the freezing point of water (D'Amico *et al*, 2002). One striking example is *Moritella profunda*, a deep-sea bacterium that cannot survive at temperatures above 10°C and whose optimal growth temperature is 2°C (Xu *et al*, 2003). Unlike high temperatures, which cause improper folding of proteins, low temperatures have no global effect on protein folding. Instead, cold temperature induces gelling of membrane bilayers and reduces membrane fluidity (Marr and Ingraham, 1962), disrupts protein synthesis by stabilising RNA secondary structures (Broeze *et al*, 1978) and impedes enzyme activity (Siddiqui *et al*, 2004) by stabilising the necessary flexibility in and around the active site (Merz *et al*, 2000). These effects combine to slow down all metabolic pathways in the cell, which consequently inhibits cell growth and division (Ng *et al*, 1962).

Mesophiles can partially circumvent the effects of cold temperature by inducing a cold shock response and expressing cold shock proteins (Csps) that are involved in effecting this stress response (Gualerzi *et al*, 2003). The most abundant and well-studied Csps belong to the CspA family of proteins (Phadtare *et al*, 1999). In *E. coli*, CspA can bind single-stranded DNA (Newkirk *et al*, 1994) but functions mainly as an RNA chaperone and can enhance transcription by destabilising RNA (Jiang *et al*, 1997). Psychrophiles also overexpress Csps and additionally express several cold acclimation proteins (Caps), which are related to mesophilic cold shock proteins (Michel *et al*, 1997). Caps can destabilise RNA secondary structure and enhance initiation of protein synthesis (D'Amico *et al*, 2006). Although Csps in both mesophiles and psychrophiles are only transiently expressed following cold shock,

the psychrophile-specific Caps are expressed during long-term exposure to cold temperatures (Berger *et al*, 1997) and this may account for the ability of psychrophilic organisms to grow for prolonged periods in the cold.

Psychrophiles have adapted several other mechanisms to be able to grow at cold temperatures. Although they most often have no sequence similarity to known mesophilic enzymes (Ryu *et al*, 2005), cold-adapted enzymes often have similar tertiary structures to those of their mesophilic homologues but with additional flexibility around their catalytic sites (Russell *et al*, 1998; Tsuruta *et al*, 2005), which is thought to account for their increased activity at cold temperature (Merz *et al*, 2000). This is supported by the observation that malate dehydrogenase from the psychrophilic *Aquaspirillum arcticum*, which has increased flexibility of active site residues compared to mesophilic or thermophilic homologues, is functional at temperatures as low as 4°C but is much less stable than these homologues at higher temperatures (Kim *et al*, 1999), suggesting that balancing stability and flexibility is crucial for the optimal activity of enzymes in psychrophiles and psychrotolerant organisms (Bae and Phillips, 2004). Similar results have been observed with the DNA ligase N-terminal domain of the psychrophile *Pseudoalteromonas haloplanktis*, which is more flexible than that of its mesophilic homologues (Georlette *et al*, 2000). Further evidence comes from site-directed mutagenesis studies, which have revealed that the presence of fewer proline residues (Huston *et al*, 2004) and additional arginine, histidine and aspartate residues (Tsuruta *et al*, 2004; Kawakami *et al*, 2007) in the active site of cold-adapted enzymes confer additional flexibility in and around the active site and are essential for enzymatic activity at cold temperatures. Another interesting adaptation is the ability of psychrophiles to produce high concentrations

of exopolysaccharides (Krembs *et al*, 2002; Nichols *et al*, 2004), which are thought to act as cryoprotectants by increasing water retention (Nichols *et al*, 2005) and creating a stable environment for secreted enzymes (Huston *et al*, 2004).

The most well-understood mechanism of cold adaptation is the ability of bacteria to alter the fatty acid composition of their lipid membranes to increase its fluidity and prevent gelling, a process referred to as homeoviscous adaptation (Sinensky, 1974). These modifications include one or more of the following: an increase in fatty acid unsaturation, an increase in average chain length and an increase in branching (Russell, 1997), the most common being an increase in the ratios of *cis*-vaccenate (18:1) and palmitoleate (16:1) to palmitate (16:0) (Figure 1-2). Phospholipids containing branched and unsaturated fatty acids have lower melting temperatures than those with saturated fatty acids, are more flexible as a result and therefore confer more fluidity to the cell membrane (Willecke and Pardee, 1971). These changes can occur within seconds and are not dependent on any new protein synthesis, suggesting that the necessary enzymes are present under all growth conditions but become active only when required (Garwin and Cronan, 1980). Modifications in fatty acid composition in response to temperature changes are also present in mesophiles (Wada *et al*, 1990), though it is possible that the cold adaptation of psychrophile enzymes allows them to modify their membranes at lower temperatures. Some psychrophiles can increase the number of specific membrane proteins and pigments that reduce membrane gelling (Chintalapati *et al*, 2004), although this mechanism is not well understood and other psychrophiles seem to achieve the same result by decreasing synthesis of these pigments (Fong *et al*, 2001).

Many of the products and enzymes expressed by psychrophiles have

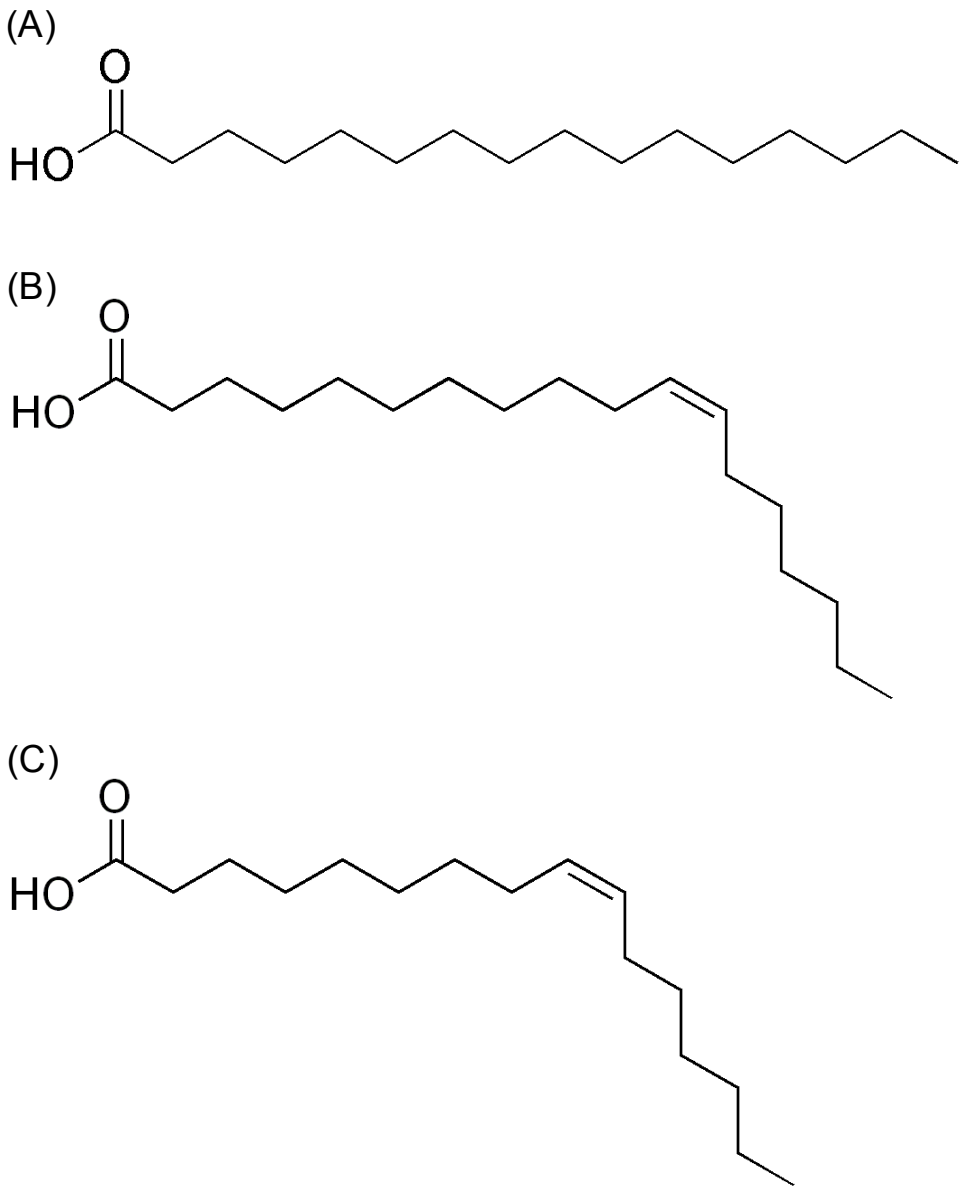


Figure 1-2. Some fatty acids involved in cold temperature adaptation. Psychrophiles can increase the fluidity of their cell membranes by converting palmitate (A) to *cis*-vaccenate (B) and palmitoleate (C).

potentially important biotechnological and industrial applications. The unsaturated fatty acids from marine psychrophiles could be used as lipid supplements in food (Russell, 1998). Psychrophilic enzymes have a large number of applications in many areas. Lipases and proteases can be used as detergent additives in cold water washing, cellulases could be used to increase durability and softness of cotton without damaging it with heat, and many enzymes can be used for bioremediation of cold environments to chelate heavy metals and break down biopolymers, toxic and aromatic compounds (Gerday *et al*, 2000). Cold-adapted enzymes such as DNA ligases could also be used in molecular biology (Georlette *et al*, 2000), since they tend to have higher catalytic activity and help in minimising unwanted reactions that usually occur at the higher temperatures (Cavicchioli *et al*, 2002).

1.1.2. High pressure and piezophiles

Piezophiles (Yayanos, 1995) are organisms that grow optimally at high pressure and induce a stress response at atmospheric pressure (Figure 1-3). In piezosensitive microorganisms, increased pressure (over 30MPa) leads to growth inhibition and accelerated death (Zobell and Johnson, 1949) as cells filament, since they can elongate but are unable to divide (Zobell and Cobet, 1962). Although one study claimed to observe viable *E. coli* cells at pressures over 1GPa (Sharma *et al*, 2002), the methods used to assess viability were controversial (Yayanos, 2002). In studies that focus on bacterial growth and division at high pressure, the usually accepted maximal pressure at which piezosensitive bacteria are still capable of growth is 50MPa (Bartlett, 2002). Higher pressures inhibit enzyme activity (Morild, 1981) and as a result disrupt a variety of essential cellular processes including DNA

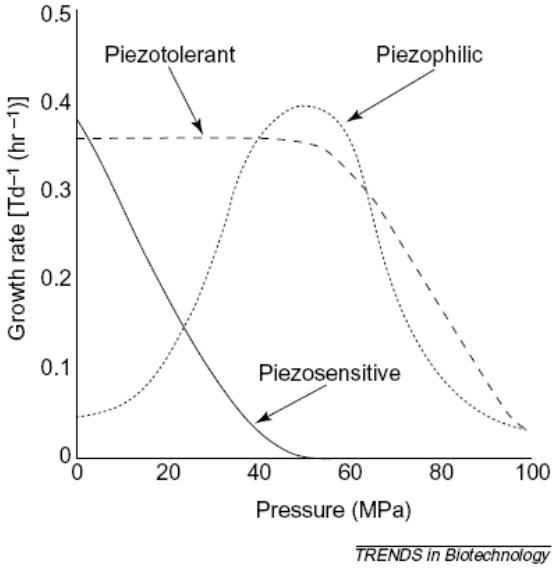


Figure 1-3. Microbial growth under pressure. Piezosensitive organisms (mesophile) grow optimally at atmospheric pressure and are stressed at higher pressures. Piezophiles grow optimally at high pressure and are stressed at lower pressures. Piezotolerant organisms grow over a larger range of pressures. (source: Abe and Horikoshi, 2001)

replication and transcription (Yayanos and Pollard, 1969), ribosome structure (Gross and Jaenicke, 1990), protein translation (Gross *et al*, 1993) and cell division (Zobell and Cobet, 1964). High pressure can also trigger a cellular pathway involving the Mrr endonuclease (Aertsen and Michiels, 2005) and this is in fact thought to be the cause behind cell filamentation at high pressure. Enhanced Mrr activity leads to double-stranded DNA damage, thereby triggering the SOS response and inhibiting polymerisation of FtsZ, which in turn leads to failed septation and filamentation (Aertsen *et al*, 2004). High pressure has the same effect as cold temperature on the cell membrane (Denich *et al*, 2003), since compression raises the gelling temperature of the membrane bilayer and reduces its fluidity (Huang *et al*, 1993). Environmental factors such as pH (Abe and Horikoshi, 1998) and nutrient availability (Marquis, 1982) can sometimes have drastic effects on the ability of microorganisms to tolerate high pressure. Interestingly, both cold and heat shock proteins are induced in *E. coli* under high pressure (Welch *et al*, 1993) and the lethal effects of high pressure can be alleviated somewhat by raising the temperature (Zobell and Oppenheimer, 1950), suggesting a close relation between the effects of pressure and those of temperature on bacterial growth.

High pressure, like cold temperature, is a condition to which deep-sea extremophiles have adapted, most notably by evolving a control system where genes are differentially regulated depending on the pressure the organism is subjected to (Nakasone *et al*, 1998). Some of the proteins whose expression is induced at high pressure include stress response proteins such as the molecular chaperones DnaK and GroEL, which may help stabilise other proteins and help restore their proper folding at high pressure (Abe *et al*, 1999). Some species of deep-sea *Shewanella benthica*

can regulate expression of different cytochrome *c* molecules depending on the pressure, although how this benefits the bacterium is not fully understood (Qureshi *et al*, 1998). Likewise, *Shewanella violaceae* will only express cytochrome *bd* at high pressure (Tamegai *et al*, 2005), even though *E. coli* uses a homologous cytochrome *bd* system under normal growth conditions at atmospheric pressure (Kita *et al*, 1984). Other studies with *Shewanella* have shown that single-strand DNA binding (SSB) proteins from piezophilic strains dissociate at higher pressure than those from piezosensitive strains (Chilukuri *et al*, 2002). The piezophilic SSB proteins share high sequence homology with their mesophilic counterparts but are much more hydrophilic, suggesting that their quaternary structure may be more stable and less susceptible to dissociation at high pressure (Chilukuri and Bartlett, 1997). Deep-sea bacteria respond to the reduced membrane fluidity induced by high pressure in the same manner they do with cold temperature, by raising the proportion of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids (DeLong and Yayanos, 1985), which suggests that homeoviscous adaptation is essential for life in the deep sea (Yano *et al*, 1997). PUFAs are of great interest to the biomedical and food industries because they have been shown in some clinical trials to reduce the risk of cardiovascular disease and cancer (Simopoulos, 1991).

Research on piezophiles is of great interest because of the potentially useful compounds that they produce, such as PUFA. Additionally, pressure is being considered as a means of sterilisation that can preserve food texture and taste better than heat sterilisation (Gould, 1996). Therefore, understanding the ability of bacteria to survive at high pressure is essential if high pressure sterilisation is to gain widespread use. Two approaches could be used to study deep-sea microorganisms,

using either metagenomics or an organism as a model system. However, an obligate piezophile is difficult to manipulate because it requires a number of pressure vessels and cannot be grown under atmospheric conditions. On the other hand, a facultative piezophile can be genetically manipulated at atmospheric pressure and then studied under high pressure. One such bacterium is *Photobacterium profundum* SS9.

1.2. *Photobacterium profundum* SS9

Photobacterium profundum SS9 is a rod-shaped, moderately piezophilic and psychrophilic γ-proteobacterium (Nogi *et al*, 1998) that was isolated from an amphipod homogenate obtained from a depth of 2.5km in the Sulu Trough (DeLong, 1986). *P. profundum* SS9 is a member of the *Vibrionaceae* and is closely related to *Vibrio cholerae* and *Vibrio fischeri* (Figure 1-4). Its genome organisation is similar to that of its *Vibrio* relatives, with two chromosomes (4Mbp and 2.2Mbp) and one large plasmid (80kbp) (Vezzi *et al*, 2005). Two other *P. profundum* strains have been isolated, the moderately piezophilic *P. profundum* DSJ4 (Nogi *et al*, 1998) and the piezosensitive *P. profundum* 3TCK (Campanaro *et al*, 2005). Interestingly *P. profundum* SS9 has a pathogenicity island that is also present in the fish pathogen *Photobacterium damselae*, in *V. cholerae* and even in *Yersinia enterocolitica* (Osorio *et al*, 2006), even though no known evidence suggests that *P. profundum* is pathogenic itself. *P. profundum* SS9 grows optimally at 28MPa and 15°C, but can grow over a range of temperatures (2-20°C) and pressures (0.1-90MPa). Even though it can grow at atmospheric pressure, *P. profundum* SS9 is considered to be a true piezophile because several of its stress response genes are activated at 0.1MPa, including *dnaK* and *groEL* (Vezzi *et al*, 2005), which interestingly are activated in *E.*

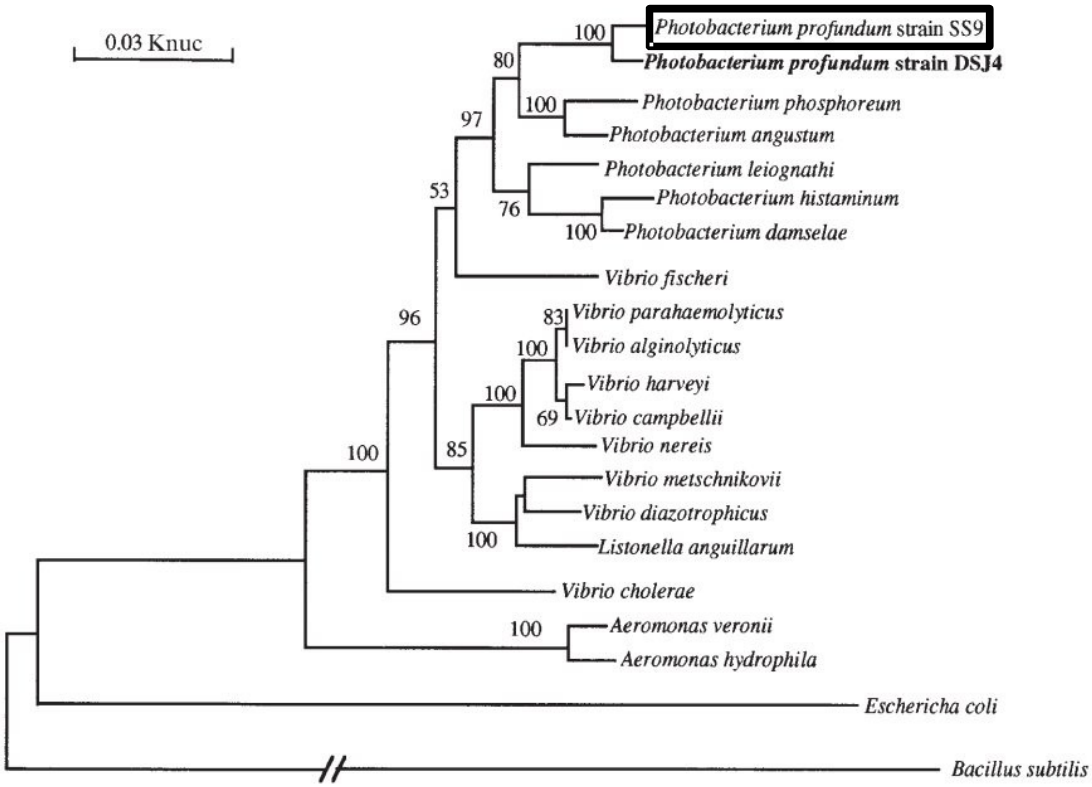


Figure 1-4. The phylogeny of *P. profundum* SS9. The phylogenetic tree shows *P. profundum* SS9 and its relation to other *Photobacterium* species and the *Vibrionaceae*. (source: Nogi *et al*, 1998)

coli at high pressures (Welch *et al*, 1993). The ability of *P. profundum* SS9 to grow over a broad range of temperatures and pressures, particularly atmospheric pressure, makes it an ideal model system to understand the molecular basis of cold and pressure adapted growth. Genetic manipulations can be carried out on the bench without killing the bacterium. Additionally, the genome is fully sequenced (Vezzi *et al*, 2005), and a set of genetic tools for mutagenesis as well as expression vectors have been developed specifically for use in *P. profundum* SS9 (Lauro *et al*, 2005), which greatly facilitates the genetic tractability of the organism.

Investigations into adaptations to the deep-sea in *P. profundum* SS9 have yielded varied results. The *fabF* gene product β-ketoacyl-acyl carrier protein synthase II (KAS II) synthesises *cis*-vaccenate, a MUFA that is required for cold temperature and high pressure growth of *P. profundum* SS9 (Allen and Bartlett, 2000). This contrasts with *E. coli* KAS II, which increases the levels of *cis*-vaccenate in response to low temperature but not high pressure (Garwin *et al*, 1980), and suggests that *P. profundum* SS9 KAS II is specifically adapted for piezophilic growth. Additionally, four genes have been identified in *P. profundum* SS9 that form the *pfaABCD* cluster involved in the biosynthesis of PUFA (Allen and Bartlett, 2002). However, unlike *Moritella* and *Shewanella* species that contain the *pfaABCDE* cluster (Orikasa *et al*, 2006), the fifth gene, *pfaE*, is not part of the cluster in *P. profundum* SS9 (Sugihara *et al*, 2008) and is in fact on Chromosome II, whereas *pfaABCD* are on Chromosome I. In contrast to other psychrophiles where increased proportion of both MUFA and PUFA are required for cold adaptation, *P. profundum* SS9 only requires higher levels of MUFA to grow at cold temperature or high pressure, even though PUFA levels also increase under these conditions (Allen

et al, 1999).

Some enzymes have been shown to have additional or modified domains that may be important in piezophilic growth of *P. profundum* SS9. Comparison of amino acid sequences in malate dehydrogenase (Mdh) from *P. profundum* SS9 and several mesophiles revealed SS9-specific substitutions in residues that are involved in either subunit or nucleotide interaction, suggesting a role for these sites in high pressure activity of the enzyme (Welch and Bartlett, 1997). RecD, a component of the RecBCD complex involved in double-stranded DNA repair and recombination in *E. coli* (Myers and Stahl. 1994), is required for growth of *P. profundum* SS9 at high pressure (Bidle and Bartlett, 1999). Interestingly, the *P. profundum* SS9 RecD protein contains an additional 64 amino acid motif that is not found in piezosensitive bacteria, which suggests this domain may confer additional activity to RecD that is involved in *P. profundum* SS9 growth at high pressure (Bidle and Bartlett, 1999). Additionally, *P. profundum* SS9 accumulates solutes at high pressure, especially β-hydroxybutyrate, and these solutes are called piezolytes because they are thought to protect protein structure and function from the effects of hydrostatic pressure (Martin *et al*, 2002).

P. profundum SS9 has also been shown to modulate the expression of certain outer membrane proteins (Omp) in response to changes in pressure (Figure 1-5). OmpH is an outer membrane porin in *P. profundum* SS9 (Bartlett *et al*, 1993) that is expressed at high pressure and not atmospheric pressure (Bartlett *et al*, 1989). Reciprocally, OmpL is another outer membrane porin (Welch and Bartlett, 1996) that is expressed at atmospheric but not high pressure (Chi and Bartlett, 1993). However the role that OmpH plays in high pressure adaptation is not fully understood, since

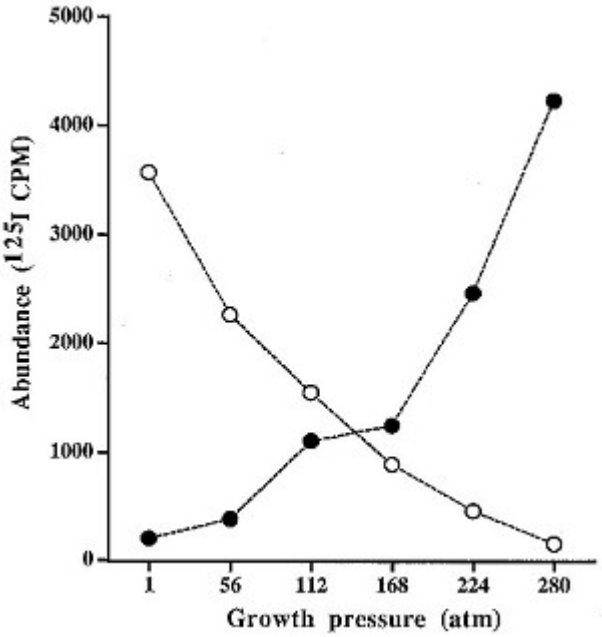


Figure 1-5. Regulation of OmpH and OmpL abundance in response to pressure. As pressure increases, OmpH abundance increases while OmpL abundance decreases. Abundance from equivalent amounts of total cell protein was quantified by gamma counting using ^{125}I-labelled antibodies. (modified from Welch and Bartlett, 1996)

the porin seems to function as a non-specific diffusion channel (Bartlett and Chi, 1994) and its expression is regulated by other environmental factors independent of pressure, including cell density, cAMP concentration, carbon starvation and catabolite repression (Bartlett and Welch, 1995). A putative *rpoE* locus with high identity to the *E. coli rpoE* gene, which encodes the alternative sigma factor σ^E that regulates the expression of stress response proteins in *E. coli* (Dartigalongue *et al*, 2001), was found to regulate the expression of outer membrane proteins and is essential for cold and pressure adapted growth in *P. profundum* SS9 (Chi and Bartlett, 1995). Expression of OmpH and OmpL is also regulated by ToxR (Welch and Bartlett, 1998), a transmembrane DNA-binding protein that plays a crucial role in the pathogenicity of other *Vibrionaceae* such as *V. cholerae* (Miller *et al*, 1987). In spite of the knowledge already gathered, much work remains to be done before a clear model for the deep-sea adaptation of *P. profundum* SS9 can be drawn.

1.2.1. The P. profundum SS9 mutant library

In order to gain further insights into genes that could play a crucial role in the cold and pressure adaptation of *P. profundum* SS9, our collaborators at the Scripps Institution of Oceanography (University of California, San Diego, USA) used SS9R, a spontaneous rifampicin-resistant *P. profundum* SS9 derivative (Chi and Bartlett, 1993), to construct a random mini-Tn*5* and mini-Tn*10* transposon mutant library of *P. profundum* SS9R (Lauro *et al*, 2008). The library was then screened for either mutants that were cold-sensitive at 0.1MPa and 4°C or for mutants that were pressure-sensitive or pressure-enhanced at 45MPa and 17°C (Figure 1-6). A total of 31 mutants were isolated from this screen (Table 1-1). Some of the mutants were

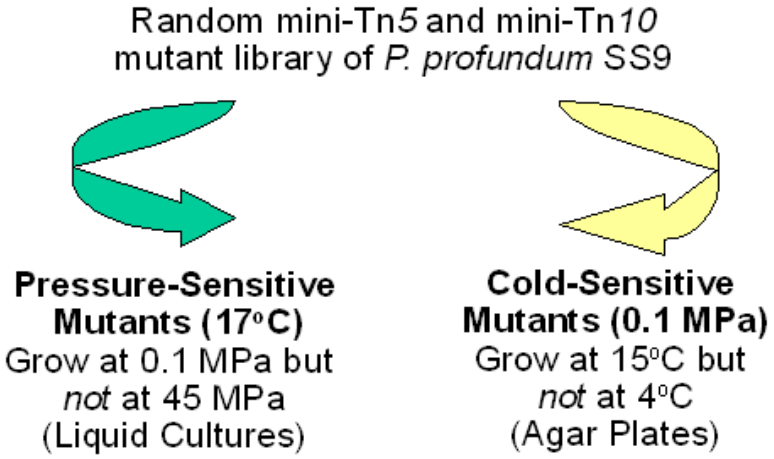


Figure 1-6. Screening for pressure and/or cold-sensitive mutants of *P. profundum*. A random transposon mutant library was constructed using mini-Tn*5* and was screened either for pressure-sensitive mutants that did not grow at 15°C and 45MPa in Marine broth or for cold-sensitive mutants that did not grow at 4°C and 0.1MPa on Marine agar.

Table 1-1. The *P. profundum* SS9 mutants (modified from Lauro *et al*, 2008)

Mutant	Gene	Annotation	Type of transposon insertions recovered in ORF	Phenotype
FL1	PBPRB2014	Transcriptional regulator; LuxR family	a-Tn*5*, b-Tn*10*	CS
FL2	PBPRB0212	ATP-dependent DEAD box RNA helicase	Tn*5*	CS
FL3	PBPRA2678	Hypothetical protein	a-Tn*5*, b-Tn*5*	CS
FL4	PBPRA3093	*rpoE* regulatory protein RseB	Tn*5*	CS, PS
FL5	PBPRA2681	Glycosyl transferase	a-Tn*5*, b-Tn*5*	CS
FL6	PBPRA1774	ATP-dependent protease; LA related	Tn*10*	CS
FL7	PBPRA2684	Hypothetical protein involved in polysaccharide biosynthesis	Tn*5*	CS
FL8	PBPRA0396	Hypothetical integral membrane protein	a-Tn*5*, b-Tn*5*	CS
FL9	PBPRA2686	Tyrosine protein kinase	Tn*5*	CS
FL10	PBPRA0428	Pyruvate kinase I	a-Tn*10*, b-Tn*10*	CS, PS
FL11	PBPRA1082	DNA-binding protein H-NS	Tn*5*	CS, PE
FL12	PBPRA0747	Suppressor protein SuhB	Tn*10*	CS, PS
FL13	PBPRA0189	Guanosine-3,5-bis(diphosphate) 3-pyrophosphohydrolase SpoT	Tn*5*	CS, PS
FL14	PBPRA2407	Periplasmic linker protein	Tn*5*	CS
FL15	PBPRA3239	Hypothetical protein	Tn*5*	CS
FL16	PBPRA2282	Conserved hypothetical protein	Tn*5*	CS
FL17	PBPRB2009	Phosphotransferase cellobiose-specific component IIc	Tn*10*	CS
FL18	PBPRA0667	Conserved hypothetical protein	Tn*10*	CS
FL19	PBPRB1941	Hypothetical protein	Tn*10*	CS
FL20	Unknown	Transposase	Tn*10*	CS
FL21	PBPRB1757	Hypothetical protein	Tn*10*	CS
FL22	PBPRB0828	Beta-hexosaminidase	Tn*10*	CS
FL23	PBPRA3229	Phosphoheptose isomerase	Tn*10*	CS, PS
FL24	PBPRA0917	Polar flagellar protein FlaJ	Tn*10*	CS
FL25	PBPRA2700	Hypothetical protein	a-Tn*5*, b-Tn*5*	CS
FL26	PBPRA0218	O-antigen ligase	Tn*5*	CS
FL27	PBPRA0674	Conserved hypothetical protein	Tn*5*	CS
FL28	PBPRA1039	SeqA protein	Tn*10*	PE
FL29	PBPRA2596	L-Asparaginase	a-Tn*5*, b-Tn*5*	PS
FL30	PBPRA2658	3-Oxoacyl-(acyl carrier protein) synthase I	Tn*5*	PS
FL31	PBPRB0001	Chromosome II replication protein RctB	Tn*5*	PS

CS: cold-sensitive. PS: pressure-sensitive. PE: pressure-enhanced

initially isolated from the high pressure screen but upon further characterisation were also found to be cold-sensitive (Lauro *et al*, 2008). A number of mutants were found to have alterations in their cell envelope, which suggests that cell envelope integrity is critical to the deep-sea adaptation of *P. profundum* SS9 (Ferguson GP, unpublished data). This study focuses on two mutants isolated from the high pressure screen, FL23 and FL28.

1.2.2. The P. profundum FL23 and FL28 mutants

The FL23 mutant was initially isolated as a pressure-sensitive mutant, then was further characterised as cold-sensitive. This mutant carries a mini-Tn*10* insertion in the *pbpra3229* gene, which is annotated as encoding a putative phosphoheptose isomerase (Lauro *et al*, 2008). The Pbpra3229 protein shares 45% identity with GmhA, the *E. coli* phosphoheptose isomerase involved in biosynthesis of the inner core of the LPS (Brooke and Valvano, 1996). However, Pbpra3229 shares 75% identity with DiaA, an *E. coli* protein involved in the timely initiation of DNA replication (Ishida *et al*, 2004)). Although the structure of Pbpra3229 has not been experimentally determined, the high homology shared by the three amino acid sequences, in addition to extensive similarities shared by the crystal structures of the *E. coli* proteins, raises two possible functions for Pbpra3229 as well as the potential involvement of DiaA or GmhA in the high pressure and cold temperature adaptation of *P. profundum* SS9.

Another mutant isolated from the library was FL28, which has a mini-Tn*10* insertion in the *pbpra1039* gene (Lauro *et al*, 2008). FL28 was initially described as pressure-enhanced because its growth at high pressure was significantly better than at

atmospheric pressure. Pbpra1039 is annotated as SeqA, a negative regulator of DNA replication (Lu *et al*, 1994), and shares 55% identity with *E. coli* SeqA at the amino acid level, suggesting the proteins are homologues.

1.3. GmhA and LPS biosynthesis

The cell envelope of Gram-negative bacteria consists of two lipid bilayers, the inner and the outer membrane (Figure 1-7A). The outer membrane is formed mostly from lipopolysaccharide (LPS), which consists of the lipid A, a sugar core and an O-antigen made of saccharide repeats (Figure 1-7B). The lipid A anchors the LPS into the outer membrane and the inner core is a short oligosaccharide that links the lipid A to the outer core and to the O-antigen if it is present (Ulmer *et al*, 2002). The completed LPS molecule (lipid A, inner and outer core and O-antigen) is referred to as the smooth LPS, while LPS molecule lacking the O-antigen is called rough LPS. The assembly pathway of the inner core is fully elucidated (Figure 1-8) and involves the biosynthesis of nucleotide-activated *glycero-manno*-heptose, which is the major subunit of the inner core of the lipopolysaccharide (Raetz and Whitfield, 2002). GmhA is a phosphoheptose isomerase that catalyses the first step in the biosynthetic pathway of *glycero-manno*-heptose (Kneidinger *et al*, 2002). G*lycero-manno*-heptose biosynthesis is not limited to Gram-negative bacteria as it is a component of the S-layer (Figure 1-8), a crystalline array of glycoproteins found in both bacteria and archaea whose overall architecture and role are thought to be similar to that of the LPS (Kneidinger *et al*, 2001). In *E. coli* K-12, which lacks O-antigen and therefore only synthesises rough LPS, *gmhA* null mutants are unable to synthesise *glycero-manno*-heptose and as a result lack the inner core entirely,

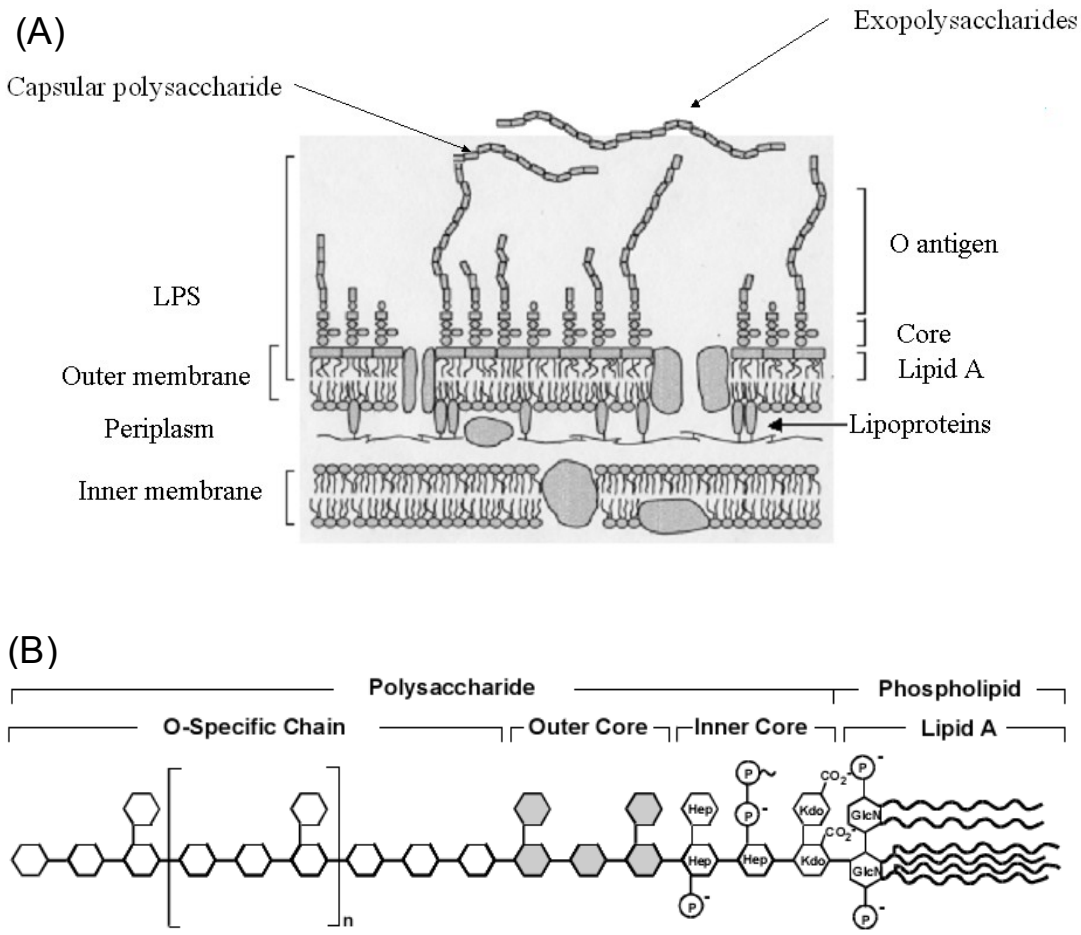


Figure 1-7. Polysaccharides in a typical Gram-negative cell envelope. A) Lipopolysaccharides are directly anchored into the outer membrane, capsular polysaccharides are attached to the outer membrane through an unknown linker, and exopolysaccharides are not attached to the membrane. B) The lipopolysaccharide consists of the lipid A, a sugar core and the O-antigen. (source: Ulmer *et al*, 2002)

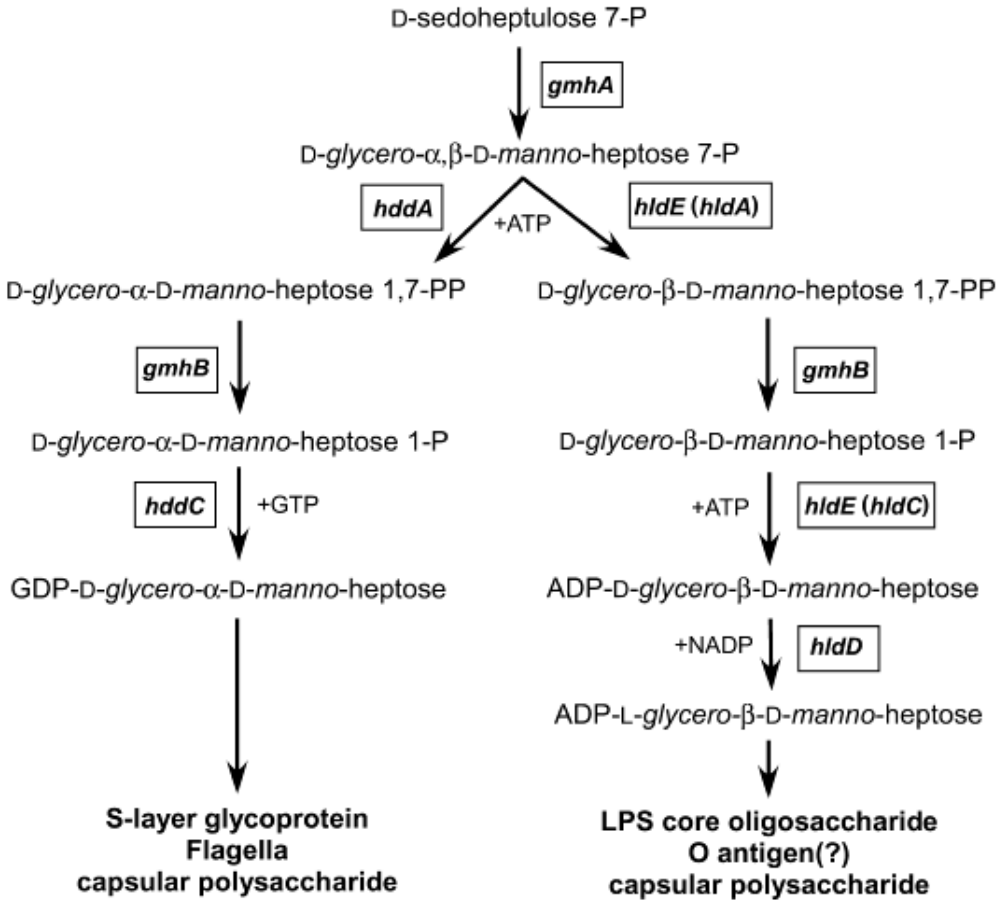


Figure 1-8. The biosynthetic pathways of nucleotide-activated *glycero-manno*-heptose. GmhA catalyses the initial isomerisation of sedoheptulose 7-P into *glycero-manno*-heptose 7-P. This molecule is used as the precursor of either the S-layer glycoprotein in Gram-positive bacteria or the LPS inner core in Gram-negative bacteria. (source: Valvano *et al*, 2002)

resulting in a truncated LPS which has the outer core attached directly to the lipid A (Figure 1-9; Brooke and Valvano, 1996). These mutants suffer from increased sensitivity to antibiotics and other membrane-damaging agents due to this alteration in their outer membrane (Tamaki *et al*, 1971). In contrast to *E. coli* K-12, the absence of the inner core in *Yersinia pestis* prevents attachment of the O-antigen to the core, even though the outer core can attach to the lipid A directly, resulting in formation of LPS lacking the O-antigen and subsequently the smooth LPS (Darby *et al*, 2005). Current evidence suggests that the LPS in *P. profundum* SS9 has an O-antigen (Allcock DJ and Ferguson GP, unpublished data), raising the possibility that a *gmhA* mutation in *P. profundum* SS9 may prevent attachment of the O-antigen to the LPS precursor.

Interestingly, a number of *P. profundum* SS9 cold and pressure sensitive mutants have insertions in genes thought to be involved in LPS or capsular polysaccharide (CPS) biosynthesis (Lauro *et al*, 2008). Some of these mutants have been shown to possess altered LPS or CPS structures, further strengthening the link between cell envelope alterations and cold temperature and high pressure adaptation (Allcock DJ and Ferguson GP, unpublished data).

1.4. DnaA and initiation of DNA replication

All known bacterial species have a copy of the highly conserved and essential gene *dnaA*, which codes for the initiator of DNA replication (Messer, 2002). The DnaA protein belongs to the AAA+ (ATPases associated with various cellular activities) superfamily, a large and diverse group involved in many different cellular processes including protein degradation, protein folding, transcriptional activation

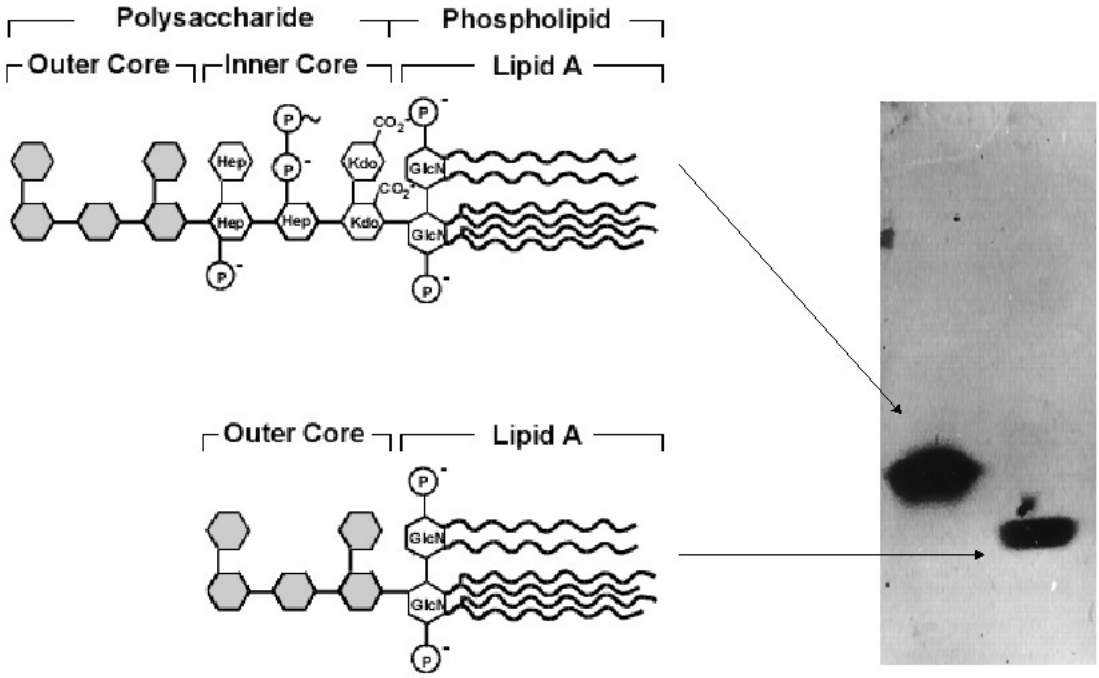


Figure 1-9. The truncated LPS in *E. coli gmhA* mutants. These mutants cannot synthesise the inner core and attach the outer core directly to the lipid A, resulting in a truncated LPS. The difference in size between the two LPS species can be visualised by SDS-PAGE and silver staining. (modified from Ulmer *et al*, 2002; Brooke and Valvano, 1996)

and DNA replication (Tucker and Sallai, 2007). AAA+ proteins are found in all three domains of life and share one common functional feature: their activity involves the ATP-driven unfolding and remodelling of nucleic acids or proteins and as a result all members share a similar structural motif formed by one or multiple copies of the ATPase domain (Ammelburg *et al*, 2006). Like other members of the AAA+ family, the active form of DnaA is ATP-DnaA, which binds to the origin of replication (*oriC*) of the chromosome and dissociates the two DNA strands, allowing DNA helicase and subsequently DNA Polymerase to load onto the unwound strands and proceed with replication (Messer, 2002). DnaA also regulates initiation of replication of many *E. coli* plasmids in conjunction with plasmid-encoded initiation proteins (Del Solar *et al*, 1998).

In all bacteria that possess a single chromosome, the origin of replication consists of an array of DnaA-boxes, an AT-rich region and a series of GATC sites (Figure 1-10). The DnaA-box is a 9-bp sequence 5′-TTATNCACA-3' (where N can be any nucleotide) that is recognised by DnaA (Ogasawara *et al*, 1985). The AT-rich region contains additional 6 bp DnaA-binding sites with the consensus sequence 5′-AGatct-3' (Speck *et al*, 1999). In *E. coli*, *oriC* is 260 bp long and contains five DnaA-boxes (Fuller *et al*, 1984).

Initiation of chromosome replication has been extensively studied in *E. coli* and the major steps in the pathway have been fully elucidated (Figure 1-11). ATP-DnaA monomers bind to the five DnaA boxes, recruiting additional ATP-DnaA molecules to the sequences between the DnaA-boxes and to the AT-rich region (Speck and Messer, 2001). Both ADP- and ATP-bound forms of DnaA can bind to the DnaA boxes, but ATP increases the affinity of DnaA for the boxes and is

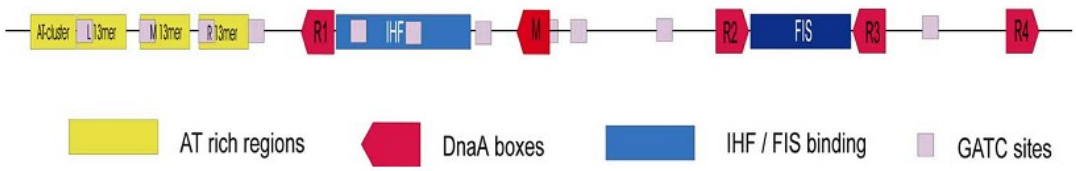


Figure 1-10. The *E. coli* origin or replication. Schematic of *oriC* showing the five DnaA boxes, the AT-rich region and the eleven GATC sites. The DnaA boxes are the initial binding sites of DnaA, though additional DnaA molecules can bind elsewhere within *oriC*. The AT-rich region is the site of the initial unwinding. The GATC sites are targeted by Dam methylstransferase and are involved in the negative regulation of initiation by SeqA. IHF and FIS are accessory proteins whose role in DNA replication is not fully understood. (modified from Messer, 2002)

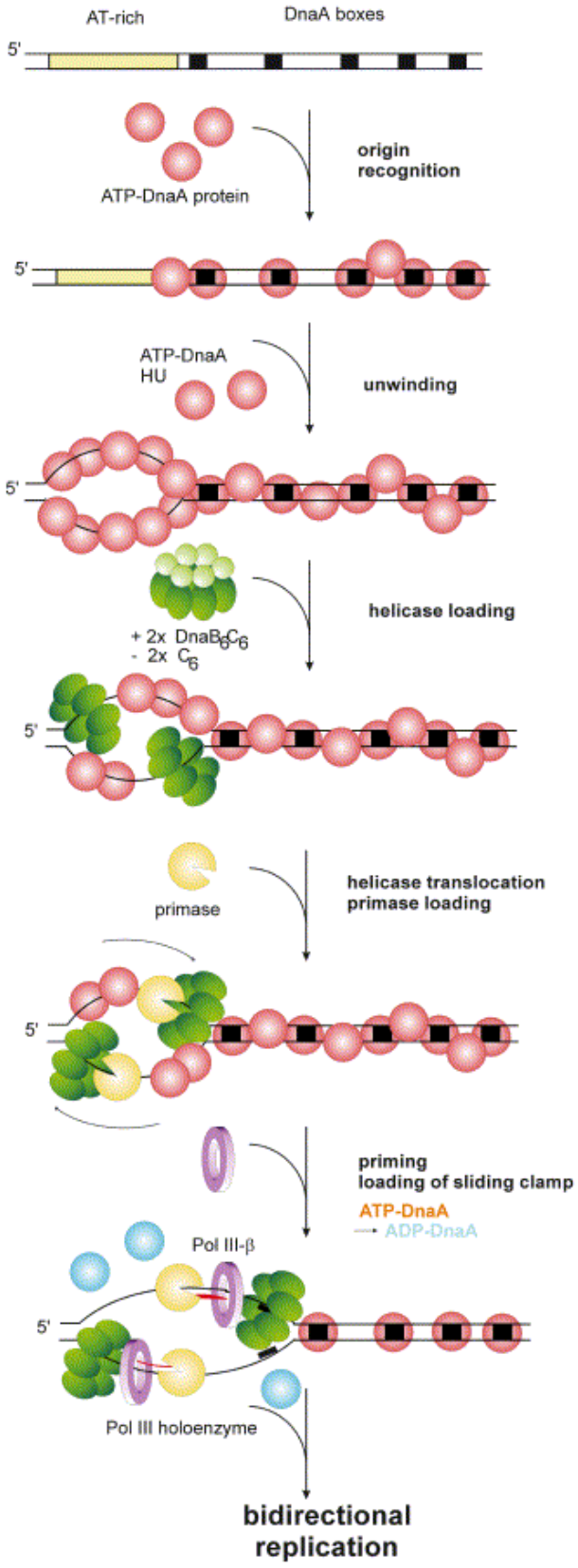


Figure 1-11. Initiation of chromosome replication in *E. coli.* Binding of DnaA to the DnaA boxes recruits additional DnaA molecules to *oriC*, resulting in the unwinding of the AT-rich region. DnaB helicase is then loaded, followed by DnaG primase and DNA polymerase III. (source: Messer, 2002)

absolutely required for binding to the AT-rich region (Speck *et al*, 1999). The DnaA proteins homo-oligomerise to form the core of the replisome (Funnell *et al*, 1987), the nucleoprotein complex that will eventually consist of all the components involved in initiation of replication at the origin. Binding of ATP-DnaA to the AT-rich region unwinds the DNA strands at this region and stabilises the single-stranded DNA (Speck and Messer, 2001). The open conformation allows loading of the DnaB helicase onto the unwound region by the helicase loader DnaC, which then immediately leaves the replisome (Carr and Kaguni, 2002). DnaB helicase further unwinds the DNA, allowing DnaG primase and DNA Polymerase III to complete the replisome and initiate DNA synthesis (Hiasa and Marians, 1999). Loading of DNA Polymerase III activates the ATP hydrolysis by DnaA and the resulting inactive ADP-DnaA is unable to initiate further rounds of replication (Katayama and Sekimizu, 1999). ADP-DnaA relocalises to the cell membrane (Newman and Crooke, 2000), where the acidic phospholipid cardiolipin can rejuvenate the DnaA molecules by displacing ADP and restoring the inactive form of DnaA to full activity by regenerating the ATP-bound form (Sekimizu and Kornberg, 1988).

In addition to being the initiator of DNA replication, DnaA can act as a transcription factor by binding to DnaA-boxes within the promoter region of genes. DnaA can activate the promoter of *polA*, which codes for DNA Polymerase I involved in DNA replication and repair (Quiñones *et al*, 1997). The promoter of the *dnaA* gene itself has a DnaA-box (Hansen *et al*, 1987) and binding of DnaA to the *dnaA* promoter represses the expression of the gene (Messer and Weigel, 1997). This complex mechanism allows DnaA to autoregulate its own expression and provides control over the timing of initiation of replication. A number of other mechanisms

are available to ensure initiation of replication occurs only once per cell cycle. One such mechanism, the regulation inactivation of DnaA (RIDA), involves the hydrolysis of ATP to produce inactive ADP-DnaA, as discussed above. The *datA* locus, which has much higher affinity for DnaA than *oriC*, is thought to sequester DnaA away from *oriC* (Kitagawa *et al*, 1996). Deleting *datA* leads to overinitiation of replication whereas introducing additional copies *of datA* on a plasmid completely blocks replication (Kitagawa *et al*, 1998), further strengthening the role that titration of DnaA to *datA* plays in controlling the timing of initiation of DNA replication. Two other important mechanisms involve the replication regulators DiaA and SeqA.

1.4.1. SeqA and negative regulation of DNA replication

SeqA is a negative regulator of DNA replication that sequesters the origin of replication and prevents binding of DnaA to the origin (Lu *et al*, 1994). Modulation of replication by SeqA is dependent on the palindromic GATC site (Figure 1-10), which is repeated eleven times within the origin of replication and is a target for Dam (DNA adenine methylase) methyltransferase (Brooks *et al*, 1983). Dam methylates the adenine of GATC sites scattered across the chromosome to control gene expression, but the sites within *oriC* are distinct from the others because they are hemimethylated shortly after replication (Messer and Noyer-Weidner, 1988). Hemimethylation of the GATC sites just after replication inhibits the initiation of the next round of replication, which can only occur after the conversion of the GATC sites to a fully methylated state (Bakker and Smith, 1989). This inhibition is due to SeqA, which binds with very high affinity to hemimethylated DNA and much less so to fully methylated DNA (Slater *et al*, 1995). The binding of SeqA to

hemimethylated GATC sequences recruits additional SeqA molecules, which spread across *oriC* by cooperative binding and compete with DnaA for binding to the DnaA-boxes (Taghbalout *et al*, 2000). Interestingly, SeqA can displace DnaA bound to hemimethylated *oriC* but not fully methylated *oriC*, which may be due to the lower affinity SeqA has for fully methylated DNA. SeqA also inhibits re-initiation of replication by binding to the promoter region of *dnaA* and suppressing transcription of the gene (Campbell and Kleckner, 1990). The inhibitory effects of SeqA on *oriC* and *dnaA* expression are maintained until Dam re-methylates those sites, although the exact mechanism by which Dam displaces SeqA from the GATC sites is not fully understood (Nievera *et al*, 2006). Once the sites are fully methylated SeqA loses much of its affinity and DnaA can bind to *oriC* and initiate the next round of replication.

1.4.2. DiaA and timely initiation of DNA replication

One newly-discovered regulator of DNA replication in *E. coli* is the DnaA initiator-associating factor DiaA. In *E. coli*, *diaA* is the third gene in a putative operon. The three other genes, *yraM*, *yraN* and *yraP*, have no known function in any organism. The *diaA* gene was isolated as an intergenic suppressor of *dnaAcos*, a cold-sensitive allele of *dnaA* (Ishida *et al*, 2004). The *dnaAcos* mutation itself is an intragenic suppression of *dnaAts*46, a heat-sensitive point mutation coding for a protein that functions at 30°C but is unable to initiate replication at 42°C (Braun *et al*, 1987). The additional point mutations in *dnaAcos* inverts the phenotype by making the protein drastically overinitiate DNA replication at lower temperatures, resulting in cell division arrest and enabling cells to grow at 42°C but not 30°C

(Kellenberger-Gujer et al, 1978). *E. coli* DiaA forms a homotetramer that interacts with DnaA bound to the origin of replication and enhances the initiation of chromosome replication by stimulating the specific binding of ATP-DnaA to the *oriC* DnaA-boxes (Keyamura *et al*, 2007). The formation of tetramers is essential for the stimulation of DnaA assembly on *oriC* and each tetramer can bind multiple DnaA molecules, raising the possibility that DiaA promotes the formation of nucleation sites for the recruitment and binding of additional DnaA molecules to the origin of replication (Figure 1-12). Inactivation of *diaA* inhibits initiation and as a result disrupts timely initiation of DNA replication (Ishida *et al*, 2004). The inhibition of initiation in the *dnaAcos* background compensates for the overinitiation phenotype and consequently suppresses cold sensitivity and restores growth at 30°C. The inhibition of initiation due to loss of DiaA in a wild-type *dnaA* background has no effect on the ability of the mutant to grow normally as seen by the doubling time of the cells or their morphology. Likewise, overexpression of DiaA and the subsequent overinitiation also disrupts timely initiation with no noticeable result on cellular growth and morphology. DiaA is moderately conserved among Eubacteria and when found the gene is usually located within a similar gene cluster (Keyamura *et al*, 2007). One fascinating observation is that the crystal structures of DiaA and GmhA homotetramers share extensive structural similarities (Figure 1-13 A & B), even though the proposed functions of the two proteins are very different (Keyamura *et al*, 2007). *Helicobacter pylori* is the only known bacterium that uses DnaA to initiate chromosome replication but lacks any of the known replication regulators, including DiaA and SeqA (Tomb et al, 1997). However, the *H. pylori* protein HobA shares significant structural similarity with *E. coli* DiaA (Figure 1-13 C & D), even though

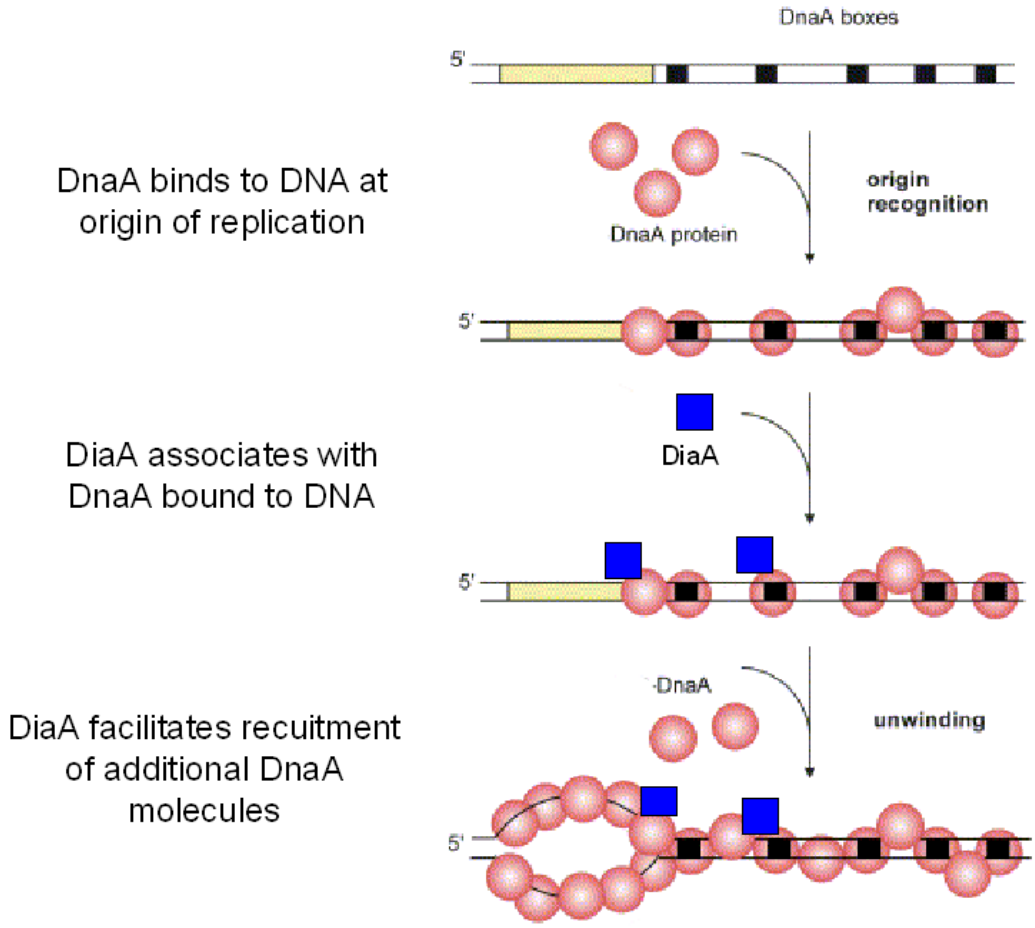


Figure 1-12. DiaA and initiation of DNA replication. DiaA forms a homotetramer that interacts with the DnaA molecules bound to the DnaA-boxes at *oriC*. DiaA then facilitates the recruitment of the additional DnaA molecules that form the core of the replisome and unwind the double-stranded DNA at the AT-rich region. (modified from Messer, 2002)

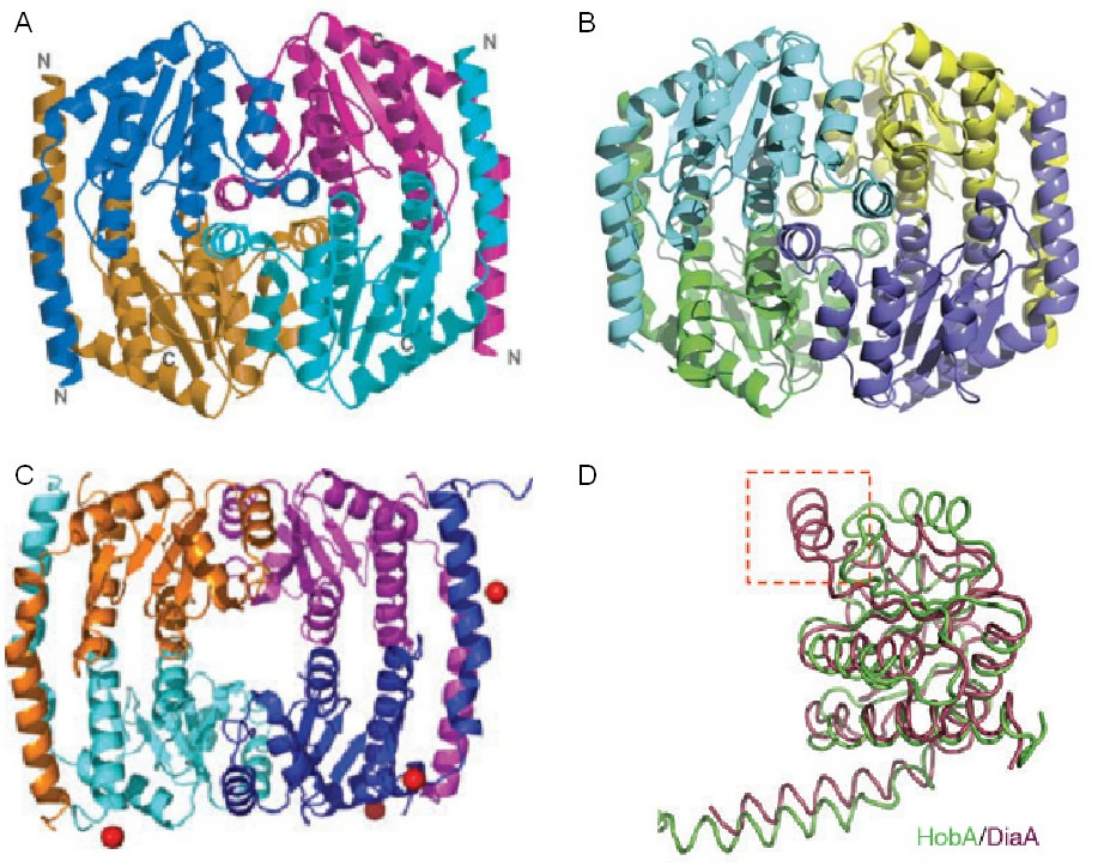


Figure 1-13. Crystal structures of GmhA, DiaA and HobA. Crystal structures of *V. cholerae* GmhA (A), *E. coli* DiaA (B) and *H. pylori* HobA (C) homotetramers showing similar structural features in the three proteins. Red balls represent calcium atoms present in the crystallisation buffer. The structural superimposition of DiaA and HobA shows the extra helix of DiaA, indicated by the dashed box (D). (modified from Seetharaman *et al*, 2006; Keyamura *et al*, 2007; Natrajan *et al*, 2007)

the proteins have no significant sequence similarity. HobA interacts with DnaA bound to *oriC* and is thought to enhance initiation of DNA replication in a similar way to DiaA (Natrajan et al, 2007).

1.4.3. DNA replication in organisms with multiple chromosomes

Although the control of DNA replication in *E. coli* is well understood, not much is known about it in other organisms, especially those with more than one chromosome. Among these, most of the studies have been limited to chromosome replication in *V. cholerae*. As mentioned earlier, most members of the *Vibrionaceae*, including *V. cholerae*, have one large and one smaller chromosome, usually referred to as chrI and chrII respectively (Heidelberg *et al*, 2000). Initiation of replication in *oriCI*, the chrI origin of replication, uses many of the same mechanisms as the *E. coli* chromosome and involves homologues of DnaA, Dam and other replication regulators, whereas initiation at *oriCII* involves a completely different system that relies on the products of the two essential genes flanking *oriCII* (Figure 1-14; Egan and Waldor, 2003). One of these genes, *rctA*, codes for an untranslated RNA whose role in initiation of replication at *oriCII* is unknown. The other gene codes for RctB, a protein that is highly conserved in the *Vibrionaceae* and binds to *oriCII* to initiate replication of chrII. A short but highly conserved DNA sequence adjacent to *oriCII* negatively regulates replication of chrII through an unknown mechanism. Interestingly, overproduction of DnaA overinitiates replication of chrI but not chrII while overproduction of RctB overinitiates replication of chrII but not chrI, further strengthening the notion that initiation of replication involves a distinct mechanism for each chromosome (Duigou *et al*, 2006). This is particularly intriguing because

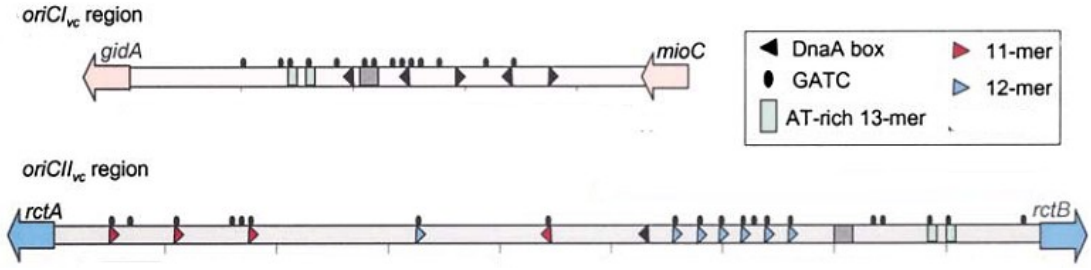


Figure 1-14. The origins of replication of the *V. cholerae* chromosomes. The origin of chrI, *oriCI*, is homologous to that of *E. coli* and utilises a similar mechanism to initiate replication of chrI. The origin of chrII is markedly different and replication of chrII requires the *rctA* and *rctB* genes flanking *oriCII*. (modified from Egan and Waldor, 2003)

initiation of replication of both chromosomes is synchronous and each one replicates only once per cell cycle, suggesting that an as yet undiscovered factor regulates the timely initiation of each chromosome and ensures synchrony (Egan *et al*, 2004).

1.5. Metagenomics

Although *P. profundum* SS9 is a useful model system for studying deep-sea adaptation, studies on a wider selection of microorganisms are desirable to expand understanding of deep-sea adaptation in multiple organisms. However, the vast majority of marine microorganisms are uncultured because they cannot be routinely grown under laboratory conditions using standard protocols and growth media (Kogure *et al*, 1979). However, one way in which to study these uncultured organisms is using the field of metagenomics.

1.5.1. The study of metagenomics

Any microorganism can be potentially cultivated if suitable culture media are designed (Button *et al*, 1993), but this method can be laborious since selecting what nutrient should be present in the medium is a trial-and-error affair. As mentioned earlier, modern molecular tools have made it possible to circumvent this problem partially by using metagenomics, or the study of genomes directly recovered from environmental samples rather than from clonal cultures (Handelsman *et al*, 1998). The totality of genes from the environmental sample is referred to as the metagenome. Although the idea of directly cloning environmental DNA was proposed as early as the mid-eighties (Pace *et al*, 1985), it wasn't until years later that the first metagenomic library was constructed (Schmidt *et al*, 1991). Constructing a

metagenomic library directly from an environmental sample bypasses laboratory culturing entirely and makes all the genes available for screening, including genes from microorganisms that have not been cultured so far. By cloning and expressing the environmental DNA in *E. coli* or other suitable hosts, novel gene products from the metagenome can be isolated.

One of the most ambitious projects dealing with environmental genomics to date has been the Sargasso Sea Whole Genome Sequence (WGS). The Sargasso Sea lies at the heart of the Bermuda Triangle and its waters are separated from the rest of the Atlantic Ocean by strong currents that completely surround the Sea (Figure 1-15), creating a region of limited nutrient availability. The Sargasso Sea WGS project generated a database of 1.2 million gene sequences from an estimated 1800 species (Venter *et al*, 2004). Analyses of the sequence dataset using bioinformatics has yielded novel insights into the evolution of selenoproteins, which incorporate the rare selenium-containing amino acid selenocysteine that is important in human health (Zhang *et al*, 2005b). Another bioinformatics study has identified and established the phylogeny of close relatives of Mimivirus, the largest DNA virus known to date (Ghedin and Claverie, 2005). While these analyses have greatly contributed to gaining further knowledge in many topics, no proteins were expressed from the Sargasso Sea metagenomic library since the goal of the project was to sequence the DNA and build a database. The next step could be to isolate and characterise novel products with pharmaceutical or biotechnological interest from marine metagenomic libraries.

Figure 1-15. The Sargasso Sea. Map showing the Sargasso Sea separated from the Atlantic Ocean by strong currents. (source: www.bibliotecapleyades.net)

1.5.2. Biotechnological applications from metagenomic libraries

Some metagenomics libraries have already been constructed, most of them from uncultured soil microorganisms. Screening these libraries has already allowed the isolation of many products with potential applications as tools in biotechnology or molecular biology. These include enzymes from uncultured bacteria such as chitinases (Cottrell *et al*, 1999), amidases (Gabor *et al*, 2004), oxidases (McDonald and Vanlerberghe, 2005), esterases (Rhee *et al*, 2005) and lipases (Henne *et al*, 2000). Additionally, a novel tetracycline-resistance mechanism has also been identified from a metagenomic library from dental plaques (Diaz-Torres *et al*, 2003), while another metagenomic library from Antarctic plankton identified an operon that could encode the biosynthetic pathway of a spectinomycin-like antibiotic (Moreira *et al*, 2004).

Of particular interest is the isolation of products that could be used directly in medical applications. Violacein is a broad-spectrum anti-bacterial pigment that also possesses antiviral and antitumoral properties (Lichstein and Van De Sand, 1946) and is known to be produced by *Chromobacterium violaceum* (August *et al*, 2000), but screening a soil metagenomic library allowed the isolation of a homologue of violacein with identical properties produced by an uncultured bacterium (Brady *et al*, 2001). Another soil metagenomic library screen led to the identification of a single protein with no similarity to known sequences and that conferred specific antibacterial activity (Rondon *et al*, 2000). In another study, two novel broad-spectrum antimicrobial agents were isolated from a soil metagenomic library and were termed turbomycin A and B (Gillespie *et al*, 2002). Interestingly, these turbomycins were the product of a chimeric pathway, where genes from the *E. coli*

host encode the necessary pathway to provide indole as a precursor and the metagenomic DNA codes for the enzymes that convert the indole molecule into active turbomycin (Gillespie *et al*, 2002).

Perhaps the most interesting contribution from metagenomics to date arises from the discovery of proteorhodopsins (Beja *et al*, 2000). Rhodopsins are complexes of the chromophore retinal bound to a light-absorbing pigment. Although rhodopsins are found in both the *Eukarya* and *Archaea* as two distinct protein families with similar topologies and biochemical activities (Lanyi *et al*, 1998), no homologues of rhodopsins had been found in any cultured *Eubacteria*. Using a marine metagenomic library, Beja and colleagues isolated a putative rhodopsin gene in the vicinity of a 16S rRNA operon (Figure 1-16). Sequence analysis of the rRNA genes classified the organism as a proteobacterium, and biochemical studies showed the rhodopsin could bind retinal to form a light-driven proton pump (Beja *et al*, 2000). This novel rhodopsin, proteorhodopsin, did not belong to any of the previously identified families of rhodopsins. Analysis of the Sargasso Sea WGS revealed the presence of these proteorhodopsins in a large number of uncultured *Eubacteria* (Venter *et al*, 2004). This research overturned the presumption that rhodopsins were exclusive to the *Eukarya* and *Archaea* to the exclusion of *Eubacteria*.

However the vast majority of metagenomic studies so far has been limited to soil metagenomic libraries, and the largest marine metagenomic project, the Sargasso Sea WGS, aimed at creating a sequence database and not at identifying novel materials. Since different habitats like the soil and marine environments impose radically different stresses on microorganisms, it is expected that these microbes

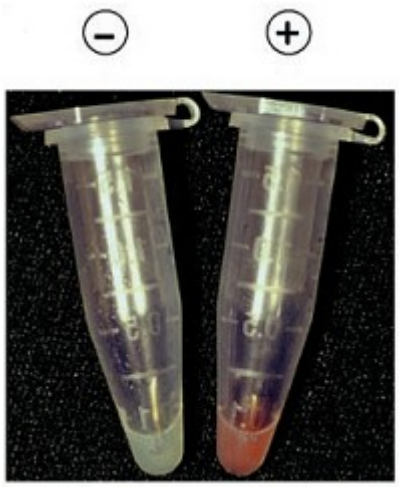


Figure 1-16. The bacterial proteorhodopsin. *E. coli* cell suspension expressing proteorhodopsin (+) compared to control cells (-). Binding of retinal to proteorhodopsin results in the characteristic red colour. (source: Beja *et al*, 2000)

would develop different strategies and components for coping with stress in each environment. This raises the possibility that many novel products of biotechnological, industrial or pharmaceutical interest could be isolated from marine metagenomic libraries. Additionally, a deep-sea metagenomic library could be used to isolate genes or clusters that could confer increased resistance to cold temperature and/or high pressure on a mesophilic host such as *E. coli*.

1.6. Aims of PhD project

The overall aim of this PhD is to understand more about pressure and cold adapted growth in marine and deep sea microorganisms.

The specific aims of the project were:

Aim 1: To further characterise the FL23 mutant, identify the function of Pbpra3229 and investigate its role in high pressure adaptation in *P. profundum* SS9.

Aim 2: To further characterise the FL28 mutant, confirm that Pbpra1039 is a homologue of *E. coli* SeqA and investigate its role in the pressure-enhanced growth of *P. profundum* SS9.

Aim 3: To identify novel polysaccharides and genes involved in cold and pressure adaptation in uncultured marine microorganisms using metagenomics.

This research was funded in part by the Leverhulme Trust, a charity that funds research into the development of novel materials. Identifying novel polysaccharides from the metagenomic library could therefore help in advancing the Leverhulme Trust's mission.

Chapter 2

Materials and Methods

2.1. General microbiology

2.1.1. Bacterial strains and plasmids

The bacterial strains used in this study are described in Table 2-1. *P. profundum* SS9R is a spontaneous rifampicin-resistant mutant of wild-type *P. profundum* SS9. *E. coli* EPI300 was used as the host strain for the pCC1FOS plasmid. For all other plasmids, *E. coli* DH5α was used as the host because the *recA* mutation eliminates recombination between cloned genes and the chromosome.

Plasmid vectors used are shown in Table 2-2. The pCC1FOS fosmid is a single-copy vector that can only be induced to high copy number in the *E. coli* EPI300 strain by addition of arabinose (0.01% w/v) to the growth medium (Khlebnikov *et al*, 2002). The *P. profundum* SS9 expression plasmid pFL190 is a broad host range vector carrying a P_{BAD} promoter that drives transcription of the cloned genes when arabinose is added to the medium (Figure 2-1 A). The pEE3 suicide vector does not replicate in *P. profundum* SS9 and allows TA cloning and blue/white selection (Figure 2-1 B). *E. coli* MM294A harbouring the plasmid pRK2013 was used as the helper strain in triparental mating experiments. Strains were stored long term by adding 10% (v/v) of either DMSO (*E. coli*) or glycerol (*P. profundum* SS9), and cultures were then frozen at -80°C.

Table 2-1. Bacterial strains used in this study

Strain	*Description*	*Reference*
P. profundum		
SS9	Wild-type isolate	DeLong, 1986
SS9R	Spontaneous SS9 mutant, Rif^R	Chi and Bartlett, 1993
FL23	SS9R *pbpra3229*::Tn*10*, Rif^R Kan^R	Lauro *et al*, 2008
FL28	SS9R *pbpra1039*::Tn*10*, Rif^R Kan^R	Lauro *et al*, 2008
FL31	SS9R *pbprb0001*::Tn*10*, Rif^R Kan^R	Lauro *et al*, 2008
E. coli		
MG1655	F^- λ^- *rph-1*	Guyer *et al*, 1981
DH5α	F^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(rk^- mk^+) *phoA supE*44 λ^- *thi*-1 *gyrA*96 *relA*1	Hanahan, 1983
EPI300	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lac*X74 *recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*)7697 *galU galK* λ^- *rpsL nupG trfA tonA dhfr*	Epicentre
TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lac*X74 *deoR recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*)7697 *galU galK rpsL nupG*, Str^R	Invitrogen
MM294A	*pro*-82 *thi*-1 *endA*1 *hsdR*17 *supE*44	Finan *et al*, 1986
X705	F^- *leu*-4 Φ^R *arg*-35 $T6^R$ λ^-, Str^R	Brooke and Valvano, 1996
X711	X705 Δ*gmhA*, Str^R	Brooke and Valvano, 1996
KH5402-1	*ilv thyA thr tyrA trpE*9829 *metE deo supF*6	Katayama and Kornberg, 1994
NA001	KH5402-1 *dnaAcos*	Katayama *et al*, 1997
NA026	KH5402-1 *dnaAcos diaA*::Tn*5*, Kan^R	Ishida *et al*, 2004
NA141	KH5402-1 *diaA*::Tn*5*, Kan^R	Ishida *et al*, 2004
BW25113	*rrnB3* Δ*lacZ*4787 *hsdR*514 Δ(*araBAD*)567 Δ(*rhaBAD*)568 *rph*-1	Datsenko and Wanner, 2000
JW0674	BW25113 Δ*seqA*, Kan^R	Baba *et al*, 2006
JW3116	BW25113 Δ*yraM*, Kan^R	Baba *et al*, 2006
JW3119	BW25113 Δ*yraP*, Kan^R	Baba *et al*, 2006
ZE0674	KH5402-1 x P1(JW0674), Kan^R	This study
ZE3116	KH5402-1 x P1(JW3117), Kan^R	This study
ZE3119	KH5402-1 x P1(JW3118), Kan^R	This study

R denotes antibiotic resistance

Table 2-2. Plasmids used in this study

Plasmid	*Description*	*Markers*	*Reference*
pRK2013	Helper plasmid for mating	Kan^R	Figurski and Helinski, 1979
pCC1FOS	Metagenomic library fosmid	Chl^R	Epicentre
TOPO	16S rRNA gene vector	Kan^R	Invitrogen
pFL190	Complementation plasmid	Str^R	Lauro *et al*, 2005
pEC*diaA*	pFL190 + *E.coli diaA* + 77bp upstream	Str^R	This study
p*pbpra3229*	pFL190 + *pbpra3229* + 70bp upstream	Str^R	This study
pEC*seqA*	pFL190 + *E.coli* seq*A* + bp upstream	Str^R	This study
p*pbpra1039*	pFL190 + *pbpra3229* + bp upstream	Str^R	This study
pEE3	Conjugal suicide vector	Kan^R	Lauro *et al*, 2005
pEE3-3227	pEE3 + *pbpra3227* internal fragment	Kan^R	This study
pEE3-3228	pEE3 + *pbpra3228* internal fragment	Kan^R	This study
pEE3-3230	pEE3 + *pbpra3230* internal fragment	Kan^R	This study

R denotes antibiotic resistance

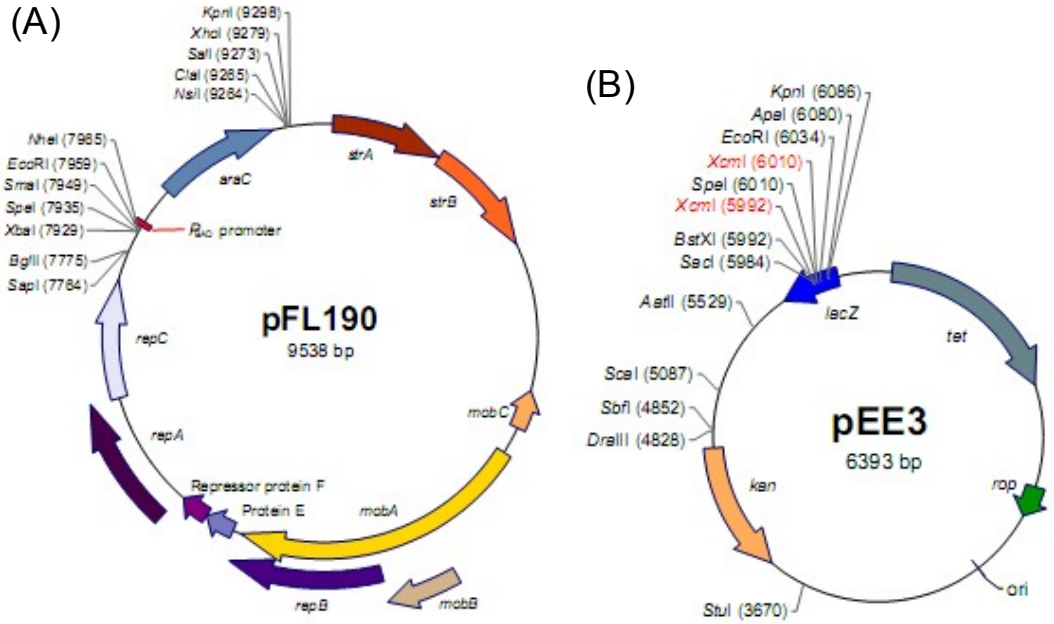


Figure 2-1. Plasmids used in *P. profundum* SS9. A: The pFL190 expression vector was specifically designed for expression in *P. profundum* SS9 and confers resistance to streptomycin. Expression of genes cloned into the multiple cloning site is controlled by an arabinose-inducible promoter (P_{BAD}). B: The pEE3 suicide vector has an XcmI restriction site in the *lacZ* gene, allowing direct TA cloning and blue/white selection. (Modified from Lauro *et al*, 2005)

2.1.2. Growth media

All *E. coli* strains were grown in Luria-Bertani (LB) broth (Sambrook *et al*, 1989). LB broth was prepared by solubilising 10g tryptone, 5g yeast extract and 10g NaCl in water to a final volume of 1 litre, adjusting the pH to 7.5 then autoclaving. To prepare LB agar, 15g of Bacto agar was added to 1 litre of broth prior to autoclaving. When growing KH5402-1 and derivatives, LB was supplemented with thymine (50μg/ml). Antibiotics were added when required (Table 2-3).

For P1 lysate experiments, top agar was prepared by solubilising 1g of Trypticase Peptone (BBL), 0.5g of NaCl and 0.65g agar in 100ml of water, autoclaving and adding $CaCl_2$ after cooling to a final concentration of 5mM.

Marine broth and Marine agar were used to grow *P. profundum* SS9 strains. For liquid cultures, 28g Marine broth (Becton and Dickinson, Difco 2216) was added to 700ml dH_2O and boiled for one minute. After cooling, the broth was filtered using grade 1, 11μm pore cellulose filters (Whatman). The medium was buffered with 100mM HEPES and adjusted to pH 7.5 with 10N NaOH. The medium volume was made up to 1 litre with dH_2O and then autoclaved. Glucose (final concentration of 20mM) was added prior to use.

To prepare 1 litre of Marine agar, Marine broth was prepared as described above but to 2x concentration by making up the final volume to 500ml only. For agar plates used at atmospheric pressure, a 2x concentrated agar solution was prepared by adding 17g of Bacto agar (Becton Dickinson) to 500ml of dH_2O. For agar plates used in high pressure experiments, the 2x concentrated agar solution was prepared by adding 25g of low gelling temperature agar (Nacalai Tesque Inc, Japan) to 500ml of dH_2O. The broth and the agar were autoclaved separately then mixed together.

Table 2-3. Antibiotics used

Antibiotic	*Final concentration (μg/ml)*		*Solvent*
	E. coli	*P. profundum SS9*	
Kanamycin	50	200	Water
Streptomycin	100	100	Water
Rifampicin	300	100	DMSO
Chloramphenicol	12.5	-	Ethanol

Glucose (20mM final concentration) and antibiotics (Table 2-3) were added just before pouring the plates. Where indicated, *P. profundum* SS9 was also grown in LB supplemented with 0.16M NaCl, 2.5mM $MgSO_4$, 2.5mM $CaCl_2$, 100mM HEPES (pH 7.5) and 20mM glucose (LB/MCSG), added after autoclaving.

2.1.3. Anaerobic pressure cultures

Unless stated otherwise, *P. profundum* SS9 strains were grown at 15°C at either 0.1 or 28MPa. To inoculate a fresh culture, 10µl of a frozen stock was added to 5ml marine broth. Liquid cultures were then transferred into sterile polyethylene transfer pipettes (4.5 ml) as described previously (Allen *et al*, 1999). After the air bubbles were removed the pipettes were heat sealed using a commercial bag sealer.

To assess the effect of pressure and/or temperature changes on growth in liquid medium, growth curves were performed using either the polyethylene pipettes or plastic bags. For pipettes, the initial cultures were diluted to an OD_{600} of 0.01 and split among a number of pipettes (typically 15 per strain per condition) and a transfer pipette was removed at each time point to measure the OD_{600}. For growth curves using bags, cultures were diluted into 200ml of fresh marine broth to an OD_{600} of 0.01, transferred into sterile plastic bags which were heat sealed and incubated at the defined conditions. To determine growth, the bags were punctured with a syringed needle and 1ml of the culture was removed before the bags were re-sealed and returned to their defined condition.

The pressure vessel (Figure 2-2) has a 3.5 litre capacity and was pressurised with water using a hydraulic pump, equipped with quick-connect fittings for rapid decompression and recompression. The internal temperature was maintained by a

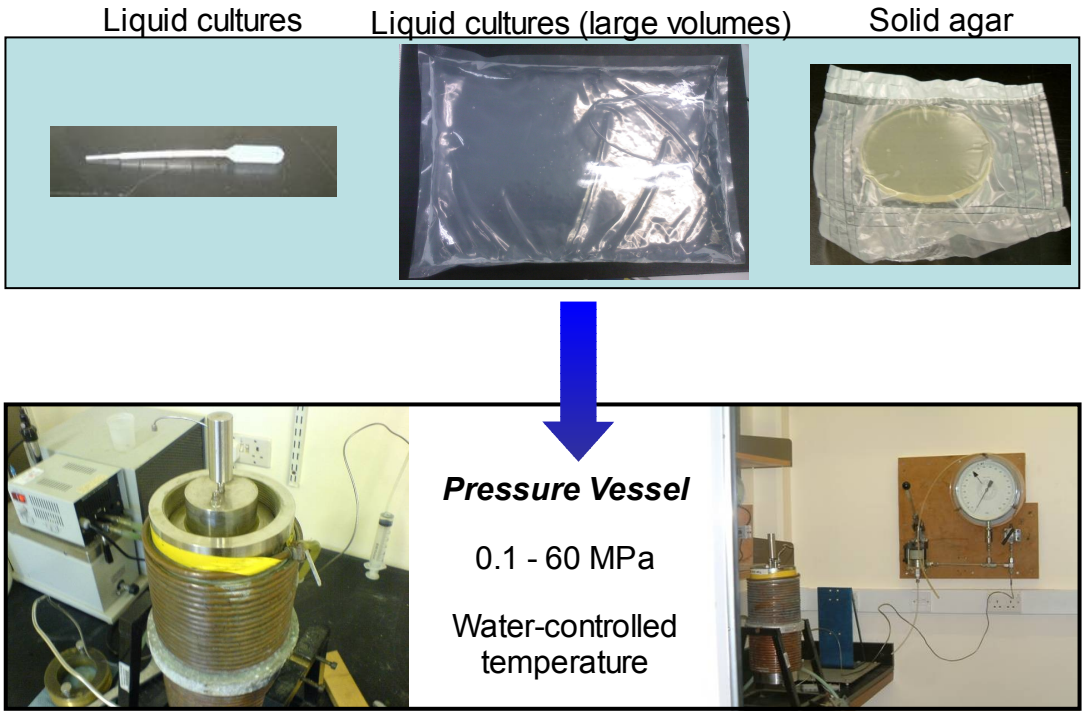


Figure 2-2. The hydrostatic pressure vessel. Liquid cultures are sealed in plastic pipettes (small volume) or bags (large volume), agar plates are sealed inside the bag. The vessel is connected to pump that can pressurise it to 60MPa and a circulating water bath that maintains vessel temperature via an outer jacket. The vessel is filled with water and has a 3.5l capacity. Samples are placed directly into the water.

circulating water jacket connected to a refrigerated circulating water bath. Pressurisation was achieved in less than 5 minutes and depressurisation in less than 2 minutes.

2.1.4. Growth on solid medium

To analyse the growth of a bacterial strain on agar, a culture was diluted in either LB or Marine broth (for *E. coli* and *P. profundum* SS9 strains respectively) to an OD_{600} of 0.2, serially diluted, then 10µl of each dilution (including the initial one) were spotted on agar and incubated under the appropriate conditions. In early experiments, Petri dishes were used, each dilution (10^0 to 10^{-6}) was spotted in triplicate and only one strain was spotted per plate (Figure 2-3 A). However, in later experiments square plates (10^0 to 10^{-5} only) were used to compare multiple strains (Figure 2-3 B).

P. profundum SS9 growth on agar at high pressure was analysed by modification of a previously published protocol (Masui and Kato, 1999). High pressure liquid cultures (grown anaerobically in the ethylene transfer pipettes, as described in section 2.1.3) were diluted in Marine broth to an OD_{600} of 0.8, serially diluted (10^{-1} to 10^{-5}), spotted onto low gelling temperature agar plates (Section 2.1.2) and incubated at 15°C for 2 hours. Following incubation, the plates were placed on ice and overlaid with 15ml of low gelling temperature agar poured directly on top of the spotted cultures. The agar "sandwich" was removed from the Petri dish and placed into sterile plastic pouches (The Vacuum Pouch Ltd, UK). The film pouch was heat sealed along the circumference of the agar, keeping enough distance as not to burn the agar but ensuring that as little air as possible remained in the bag, then

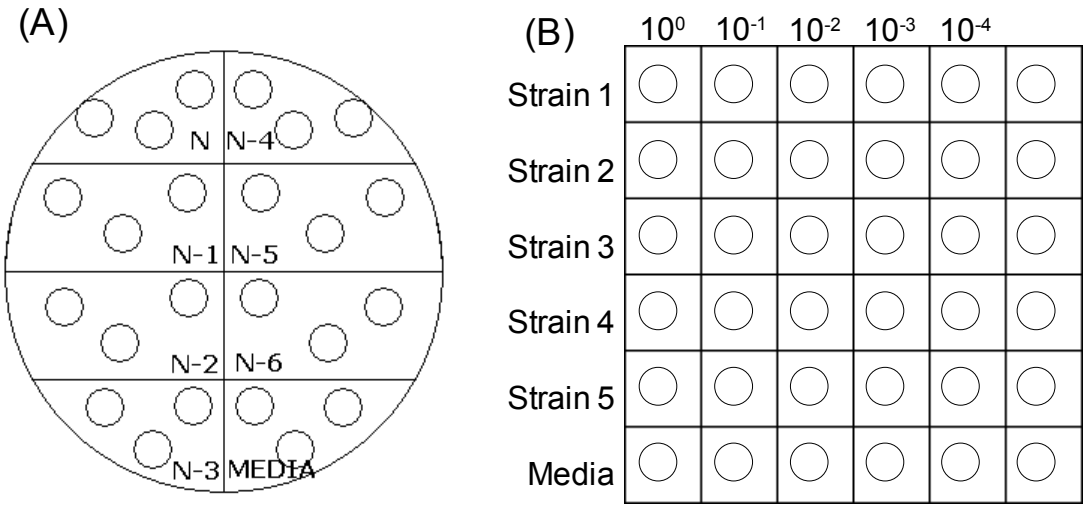


Figure 2-3. Assessing colony forming ability. A: Spotting on round plates. The initial culture (N), the serial dilutions (N-x) and a media control are spotted in triplicate on agar. Only one strain is spotted per plate. B: Spotting on square plates. Serial dilutions for up to 5 strains plus media control can be spotted on the same plate for easier comparison.

incubated in the pressure vessel as defined.

2.1.5. Microscopy

For light microscopy, cultures were grown under defined conditions and spotted on glass slides. Before use the slides were coated with poly-L-lysine (0.01% w/v) for 5 minutes then dried at 65°C for one hour. All light microscopy images were taken with a 100x oil immersion phase contrast lens on a Carl Zeiss Axioskop microscope and AxioCam using the AxioVision version 2.0 software.

Samples were treated for transmission electron microscopy by modifying a previously published protocol (Karnovsky, 1965). 1ml of the desired culture was spun down in a microfuge, the growth media was carefully removed and the pellet was washed in HEPES (100mM, pH 7.4) buffer. Cells were initially fixed in modified Karnovsky fixative (2% v/v Paraformaldehyde, 2.5% w/v Glutaraldehyde, 0.1M Sodium Cacodylate buffer, pH 7.4) for 1 hour. The fixed cells were sent to Euan James (University of Dundee) who further process the samples as follows. Cells were postfixed in aqueous Osmium Tetroxide (2% w/v) for 1 hour then dehydrated in an alcohol series and infiltrated with Spurrs resin. Samples were cut on a Leica UCT ultramicrotome to produce 90nm thick sections. These were collected on 300 mesh copper grids and stained using aqueous Uranyl acetate then counterstained with lead citrate. Imaging of the sections was undertaken on a Philips (FEI) CM 120 Biotwin TEM at 100kV. Images were taken on Kodak SO163 electron microscope film.

2.2. Polysaccharide analysis

2.2.1. LPS isolation by SDS lysis

To isolate LPS from *E. coli* and *P. profundum* SS9, cells from late exponential phase were pelleted by centrifugation at 16000*g* for 2 minutes in a microfuge. The supernatant was discarded and the pellet was resuspended in 30µl lysis buffer (1M Tris-HCl, pH 6.8, 2% w/v SDS, 4% v/v β-mercaptoethanol, 10% v/v glycerol, 0.005% w/v bromophenol blue) and boiled for 10 minutes. After cooling to room temperature, 10µl proteinase K (2.5 mg/ml stock, Sigma) was added and the samples were incubated at 60°C for 1 hour. 80µl of sample buffer (120mM Tris-HCl pH 6.8, 3% w/v SDS, 9% v/v β-mercaptoethanol, 30% v/v Glycerol, 0.03% w/v bromophenol blue) was then added. Samples were stored at -80°C.

2.2.2. LPS analysis by SDS-PAGE

The components of the gel used in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) are described in Table 2-4. The gel buffer contains 3M Tris and 0.3% (w/v) SDS, pH adjusted to 8.45.

The gel was run at 100V for 3.5 hours using 0.2M Tris-HCl, pH 8.9, as the anode buffer and 0.1M Tris, 0.1M Tricine, 0.1% w/v SDS as the cathode buffer. For sodium-m-periodate staining, the gel was fixed overnight in fixative solution (40% v/v ethanol, 5% v/v glacial acetic acid). For Alcian Blue staining, the gel was fixed overnight in Alcian Blue (0.05% w/v Alcian Blue 8GX in fixative solution).

2.2.3. Visualising LPS by Silver staining

For sodium-m-periodate staining, the fixed gel was covered with 50 ml of

Table 2-4. Preparation of gels for SDS-PAGE

Ingredients	*Concentrating gel*	*Separating gel*
H_2O	4.2ml	2.3ml
Gel buffer	1.5ml	3.3ml
Glycerol		1ml
Acryl/Bisacrylamide solution	500µl	3.3ml
10% (w/v) Ammonium persulphate	130µl	40µl
TEMED	7.3µl	4µl

oxidiser (0.7% w/v sodium-meta-periodate in fixative solution) for 5 minutes. For both staining procedures the gels were then washed 3 times for 15 minutes each in distilled water. The gels were silver stained (stain solution: 140ml water, 280µl 10N NaOH, 2ml ammonium hydroxide, 1g silver nitrate semi-dissolved in 5ml water, added drop wise) for 5 minutes, followed by 3 washes of 10 minutes each in distilled water. The gels were developed (developer: 0.02% w/v formaldehyde and 50ng/ml citrate in water) until the bands were visible, then stopped in 0.5% v/v glacial acetic acid for less than a minute. The gels were washed for 10 minutes in water then dried and photographed.

2.3. Nucleic acid manipulations

2.3.1. Agarose gel electrophoresis

Purified DNA was analysed by gel electrophoresis using 0.8% w/v agarose (Seakem LE agarose, Cambrex) in 1x TAE buffer (50x TAE buffer: 242 g/l Tris, 18.61 g/l $Na_2EDTA{\cdot}2H_2O$, 57ml glacial acetic acid). 1µl loading buffer (30% v/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF) per 5µl of sample was added before loading. The DNA ladder (New England Biolabs) used was either a 1kb ladder for plasmid DNA or a 100bp ladder for PCR products. The ladder mix was prepared by adding 2µl of the ladder to 8µl dH_2O and 2µl loading buffer, and 6µl of the ladder mix was loaded onto either side of the gel. The gel was run at 80V for ~50 minutes then stained with ethidium bromide (0.5µg/ml) for 30 minutes before being imaged.

2.3.2. Plasmid purification

Plasmids were isolated from 5ml overnight *E. coli* cultures using QIAprep Spin Miniprep Kit (Qiagen) following manufacturer's instructions. Briefly, cells were pelleted and resuspended in ice-cold resuspension buffer (including RNase). Lysis buffer was added for no more than 5 minutes before adding the neutralisation buffer. The neutralised lysate was centrifuged to pellet cell debris and the supernatant was loaded onto a purification column and centrifuged to bind the DNA to the column. Bound DNA was washed and the column was dried by centrifugation. The plasmid was eluted in two steps, each consisting of adding 30µl of Molecular Biology Reagent Water (Sigma) preheated to 65°C to the column, incubating at room temperature for 2 minutes and centrifuging. Purified plasmid DNA was stored at -20°C.

2.3.3. Fosmid copy number amplification and purification

Before the fosmid can be extracted from the EPI300 strain, it must be induced to high copy number. This was done by inoculating an overnight culture in LB broth with chloramphenicol (12.5µg/ml) at 37°C. The overnight culture was diluted 10-fold in 5ml of fresh LB containing chloramphenicol (12.5µg/ml) and arabinose (0.01% w/v) and grown at 37°C for 5 hours with vigorous shaking.

The conventional plasmid miniprep kits have a limit on the size of the DNA that can be purified, since plasmids larger than 10kb cannot be easily eluted from the columns. For fosmid clones, which are around 50kb in size, the alkaline lysis protocol from Sambrook *et al* (1989) was used. The bacterial cells were pelleted by centrifugation and resuspended in 100µl of ice-cold Solution I (50mM glucose,

25mM Tris-HCl, 10mM EDTA, pH 8.0). Cells were lysed by the addition of 200µl of freshly prepared Solution II (0.2N NaOH, 1% w/v SDS) and incubated on ice for 4 minutes. The lysate was neutralised with 150µl of ice-cold Solution III (3M potassium, 5M acetate) and incubated on ice for 5 minutes. The neutralised lysate was then centrifuged for 5 minutes at 16000*g* at 4°C to pellet the cell debris and the supernatant was transferred into a clean microfuge tube. The DNA was extracted by adding an equal volume Phenol:Chloroform:Isoamyl alcohol (25:24:1 ratio) saturated with TE at pH 8.0 (Sigma), vortexing and centrifuging for 2 minutes at 16000*g* at 4°C to separate the phases. The upper aqueous phase was transferred to a clean microfuge tube and the DNA was precipitated by adding 2 volumes of absolute ethanol, incubating at room temperature for 2 minutes and centrifuging for 5 minutes at 16000*g* at 4°C. The supernatant was discarded and the DNA pellet was rinsed with 1ml of ice-cold 70% (v/v) ethanol. After removing the ethanol, the pellet was air-dried at room temperature for 15 minutes. The DNA was dissolved in 50µl of Molecular Biology Reagent Water (Sigma) containing 20µg/ml of RNase.

2.3.4. Polymerase Chain Reaction (PCR)

Primers used in this study are shown in Table 2-5. To generate amplicons for directed insertion into plasmids, primers were designed containing restriction enzyme sites. Forward primers carried the EcoRI (G|AATTC) restriction site and reverse primers carried the XbaI (T|CTAGA) restriction site. Since the cloning vector pFL190 has a promoter but no ribosome binding site upstream of the multiple cloning site, primers were designed so that the amplicon included the entire open reading frames as well as the annotated ribosome binding sites for each gene. The

Table 2-5. Primers

Primer name	*Sequence*
pbpra3229-70F_EcoRI	5'-GTGAATTCATGTGGTCGCAATTCAAGGCC-3'
pbpra3229+618R_XbaI	5'-TTCTAGATACACCAATACTTGGCGTAGTAT-3'
ECdiaA-71F_EcoRI	5'-GTGAATTCACCGGGAATGAGGTTGAGTGGATTAAG-3'
ECdiaA+618R_XbaI	5'-GGTCTAGAGCTGGCGATAATGCCTTCATGTATTCTCC-3'
pbpra1039-57F_EcoRI	5'-GGAATTCGCTAGAGTATAACCTCGGATCA-3'
pbpra1039+561R_XbaI	5'-GCTCTAGAGCGCATATAGCCAGTTAAATAA-3'
ECseqA-81F_EcoRI	5'-GTGAATTCGGCTTGACGCTATCCGCTGC-3'
ECseqA+549_XbaI	5'-GCTCTAGACATTGCTTTGTCCTTTGTCTGCAACG-3'
190F	5'-GCGGGACCAAAGCCATGACAAAAA-3'
190R	5'-TTGTAAAACGACGGCCAGTGAGC-3'
pbpra3227+1506F	5'-CGCAAGCTCACGTGGCAACCC-3'
pbpra3230+290R	5'-GTAGGTAGAGCACGAACCTTCACC-3'
ECyraM+1674F	5'-CGGTAGCCAGAGCGGTGCAAC-3'
ECyraP+319R	5'-GGCCCTGACGAATCTCGTTATACAC-3'
pbpra3327+307F	5'-TGGCTGAGTGGCAGCTTGCAC-3'
pbpra3327+679R	5'-GACGAACAGGTCGTTGTGAATAGG-3'
pbpra3228+8F	5'-CACCCCTGCCGCTGAATAAGCG-3'
pbpra3228+328R	5'-ACTCAATCTGCTGATCGGGGCC-3'
pbpra3230+9F	5'-CGCCAAGTATTGGTGGTTGCTTTGT-3'
8F	5′-AGAGTTTGATCCTGGCTCAG-3'
1492R	5'-GGTTACCTTGTTACGACTT-3'

primers were supplied by MWG Biotech.

The Eppendorf TripleMaster polymerase was used according to the manufacturer's protocol and PCR reactions were run on an Eppendorf gradient cycler system. Two reaction mixtures were prepared as follow:

Mix 1: 1µl forward primer (25pmol), 1µl reverse primer (25pmol), 1µl of a 1:20 dilution from a -80°C frozen stock, 7µl dH_2O.

Mix 2: 1µl 10mM 4× dNTP, 2µl 10× HIFI buffer, 6.5µl dH_2O, 0.5µl TripleMaster polymerase.

Mixtures 1 and 2 were added together in a 1:1 ratio and the PCR reaction was run using the program shown in Table 2-6. After amplification the products were purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol then confirmed to be of the expected size by agarose gel electrophoresis (Section 2.3.1).

2.3.5. Restriction digestion and ligation

To clone DNA fragments into a vector, both vector and insert DNA were double-digested with the appropriate restriction enzymes (New England Biolabs). Reactions were prepared following the manufacturer's protocol using 1µl of each enzyme, 2µl of the appropriate 10x reaction buffer, 100µg/ml BSA if required and Molecular Biology Reagent Water (Sigma) to a final volume of 20µl. The reaction was incubated at the appropriate temperature for 1 hour with frequent flick mixing. The restriction enzymes were inactivated at 65°C. The restricted DNA fragment and vector were ligated in a 10µl reaction containing 100ng vector DNA, 17ng insert DNA, 1µl of 10x ligase buffer, 1µl T4 DNA ligase (Promega) and Molecular

Table 2-6. PCR program

Step	*Temperature* (°C)	*Time* (minutes:seconds)
1) Lysis	94	4:00
2) Denaturation	94	1:00
3) Annealing	64	0:50
4) Extension	72	1:00
Repeat steps 2-4 34 times		
5) Final extension	94	10:00
Hold	4	

Biology Reagent Water. The reactions were incubated at room temperature overnight. A reaction with restricted plasmid and no insert DNA was set up as a control to check for uncut or single-cut vector.

2.3.6. Cloning into pFL190 and pEE3

Table 2-7 describes the primer pairs used to amplify genes for cloning as well as the product size of the amplicon. To clones genes for complementation, amplicons included restriction sites for EcoRI at the 5' end and XbaI at the 3' end (Section 2.3.4). The amplicons and pFL190 were separately restricted with EcoRI and XbaI and the enzymes were inactivated at 65°C. Digested amplicon and plasmid were mixed and ligated overnight at 20°C. Clones were transformed into DH5α and screened for the presence of inserts by PCR using the 190F/190R primer pair (Table 2-5), which are respectively 150bp upstream and downstream of the cloning site. Positive clones were selected on the basis of a band shift from 300bp to around 1000bp (since the size of the amplicons is around 700bp). These were further confirmed to be carrying the appropriate insert by extracting the plasmid and sequencing it using the 190F primer.

To clone genes for site-directed mutagenesis, primer pairs were designed to amplify internal fragments of the genes of interest. The genes were amplified by PCR (Table 2-6) then purified using the QIAquick PCR Purification Kit (Qiagen) and confirmed to be of the expected size by agarose gel electrophoresis. Amplicons were mixed with XcmI-digested pEE3 and ligated overnight at 20°C. Clones were transformed into DH5α and plated on LB agar supplemented with X-gal (40μg/ml) and IPTG (0.1mM). White colonies were purified, the plasmid was extracted and

Table 2-7. Gene amplification for cloning

Gene	*Primer pair*	*Application*	*Product size (bp)*
pbpra3229	pbpra3229-70F_EcoRI / pbpra3229+618R_XbaI	Complementation	688
diaA	EcdiaA-71F_EcoRI / ECdiaA+618R_XbaI	Complementation	689
pbpra1039	pbpra1039-57F_EcoRI / pbpra1039+561R_XbaI	Complementation	618
seqA	EcseqA-81F_EcoRI / ECseqA+549_XbaI	Complementation	630
pbpra3227	pbpra3327+307F / pbpra3327+679R	Mutagenesis	373
pbpra3228	pbpra3228+8F / pbpra3228+328R	Mutagenesis	321
pbpra3230	pbpra3230+9F / pbpra3230+290R	Mutagenesis	282

sequenced for the presence of the correct insert.

2.3.7. DNA sequencing

Sequencing reactions were carried out by the University of Edinburgh School of Biological Sciences Sequencing Service (SBSSS) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). A 5µl reaction mixture containing 500ng of DNA and 3.2pmole of primer was sent to the SBSSS, where 3µl of sequencing buffer and 2µl of BigDye were added. The sequencing reaction was performed following the manufacturer's protocol. Sequences were confirmed by checking the chromatograms using the Chromas Lite software (Technelysium).

2.3.8. RNA extraction

To extract total RNA from bacteria cells, a culture was grown overnight in 5ml of the appropriate medium, diluted 1:50 and grown to an OD_{600} of 0.4. Cells were pelleted by centrifugation and resuspended in 1ml of Stabilization Buffer (0.1% w/v SDS, 1% v/v phenol, 19% v/v ethanol, in water). The reaction was incubated on ice for 30min then stored at -80°C. To lyse the bacteria, cells were thawed and lysosyme (40ng/ml) was added, then the cells were incubated at room temperature for 5 minutes. The RNA was then extracted using the SV Total RNA Isolation System (Promega) following the manufacturer's protocol. Briefly, RNA lysis buffer, RNA dilution buffer and 95% ethanol were added. The reaction was then transferred onto a spin column and centrifuged to allow the RNA to bind the column. The column was washed once with RNA wash solution. DNase I incubation mix was added directly on top of the column and incubated at room temperature for 15 minutes to remove all DNA. The column was washed with DNase stop solution then

washed twice with RNA wash solution. The column was then transferred onto an elution tube, the RNA was eluted in 100µl of nuclease-free water and stored at -80°C.

2.3.9. RT-PCR

One-step RT-PCR was performed using the Access RT-PCR System (Promega) and following the manufacturer's protocol. Briefly, reactions were set up by adding Reaction Buffer, dNTP mix, upstream and downstream primers, $MgSO_4$, RNA, AMV reverse transcriptase, *Tfl* polymerase and water to a final volume of 50µl. The samples were incubated at 45°C for 45 minutes to synthesise the initial cDNA strand then incubated at 94°C for 2 minutes to inactivate the reverse transcriptase. The samples were then run through a regular PCR program for 40 cycles to amplify the cDNA. Products were visualised by agarose gel electrophoresis (Section 2.3.1).

2.4. DNA introduction into bacterial cells

2.4.1. $CaCl_2$ competent cells and transformation

To make competent *E. coli* cells, a culture was grown overnight in LB with the appropriate antibiotic at 37°C unless otherwise indicated. The culture was then diluted 50-fold into 50ml of fresh LB (with antibiotic) and grown to an OD_{600} of 0.4. Cells were harvested by centrifuging at 3500*g* and 4°C for 5 minutes then resuspended in 5ml of ice-cold 100mM $CaCl_2$ and incubated at 4°C overnight. The cells were again pelleted by centrifuging at 3500*g* and 4°C for 5 minutes then carefully resuspended in 2.5ml of ice-cold 100mM $CaCl_2$ with 20% (v/v) glycerol.

Aliquots of the competent cells were stored at -80°C.

To transform the cells, 1µl of plasmid DNA was added to 50µl of competent cells. The mixture was incubated on ice for 30 minutes, heat shocked at 42°C for exactly 2 minutes then returned to ice for 10 minutes. The cells were recovered by adding 1ml of LB without antibiotics and incubating at 37°C for 1 hour. The cells were harvested by centrifugation, resuspended in 150µl LB, spread onto an LB agar plate containing the appropriate antibiotic and incubated at 37°C overnight. Transformants were purified by streaking on a fresh LB agar plate with the appropriate antibiotic. In all transformations competent cells that had undergone the procedure without any DNA added were used as a negative control.

2.4.2. Conjugation of plasmids from E. coli into P. profundum SS9 using triparental mating

The conjugation of plasmids from *E. coli* DH5α into *P. profundum* SS9 strains were performed following a previously described method with modifications (Lauro *et al*, 2008). The *P. profundum* SS9 acceptor strain was pre-grown in a polyethylene pipette (see section 2.1.3) then diluted 1:50 in 15ml of fresh Marine broth and grown overnight at 0.1MPa and 15°C. At the same time the *E. coli* donor and helper (MM294A pRK2013) strains were inoculated in 5ml of LB with the appropriate antibiotics and grown overnight. The next day, when all the cultures had reached early stationary phase, the *E. coli* cells were washed once in marine broth and concentrated 5x in Marine broth, while the *P. profundum* SS9 culture was concentrated 50x. The cultures were then mixed in a 1:1:1 ratio, plated onto dry Marine agar plates and then incubated at 20°C for 40 hours. The cells were then re-

suspended in marine broth, plated on marine agar containing streptomycin (150μg/ml) and rifampicin and then incubated at 0.1 MPa (15°C) until single colonies appeared (usually 5-10 days). Exconjugants were purified twice on Marine agar with streptomycin and rifampicin, then grown in Marine broth with streptomycin.

2.4.3. P1 lysate and transduction

To prepare a P1 lysate, the donor strain was grown in LB with antibiotic overnight. The strain was then diluted 1:50 in 20ml of fresh LB and grown at 37°C with shaking to an OD_{600} of 0.6. The cells were then pelleted and resuspended in an equal volume of LB supplemented with 25mM $CaCl_2$ and 50mM $MgSO_4$. Cells were split into six 2ml aliquots. One was the negative control (no phage) and the others had varying volumes (5, 15, 25, 50 and 100μl) of an MG1655 P1 lysate added to them. The tubes were incubated in a 37°C water bath for 30 minutes. During this time BBL Top agar was melted and allowed to cool to 55°C in a heat block. At the end of the incubation step 3ml of BBL Top agar was added to each tube and gently mixed before pouring on top of an LB agar plate supplemented with 0.5mM $CaCl_2$. The plates were incubated face-up at 37°C overnight. The next day plates were removed and observed for most confluent lysis. To harvest the P1 phage, the top agar layer was scraped using a clean sterile microscope slide (dipped in ethanol and flamed beforehand) into a sterile 50ml conical tube. Phage buffer (5mM $CaCl_2$ and 100μl chloroform in 5ml LB broth) were added and mixed by vigorous vortexing to break up the agar and the cells, then incubated at room temperature for 15 minutes. The mixture was centrifuged at 3500*g* for 15 minutes and the supernatant was

carefully transferred to another sterile tube and centrifuged again for 5 minutes to remove all traces of agar and cell debris. The supernatant was then moved to a clean vessel and 100µl of chloroform were added to inhibit bacterial growth before storage at 4°C.

To transduce the P1 lysate, the recipient strain was grown in LB overnight. The strain was then diluted 1:50 in 5ml of fresh LB and grown at 37°C with shaking to an OD_{600} of 0.6. Cells were pelleted and resuspended in 1ml of transducing medium (100mM NaCl, 5mM $CaCl_2$ and 1mM $MgCl_2$ in LB broth). Six tubes were set up: a cells-only control, a lysate-only control, and four transduction reactions with cells and various dilutions of the P1 lysate. The tubes were incubated in a 37°C water bath for 30 minutes. Cells were pelleted, washed twice with then resuspended in 1ml of LB supplemented with 10mM tri-sodium citrate and incubated in a 37°C water bath for 1 hour. The cells were harvested by centrifugation, resuspended in 150µl LB, spread onto an LB agar plate supplemented with 10mM tri-sodium citrate and the appropriate antibiotic and incubated at 37°C overnight. Transductants were purified twice by streaking on a fresh LB agar plate supplemented with 10mM tri-sodium citrate and the appropriate antibiotic.

2.5. Bioinformatics and computational analysis

2.5.1. 16S rRNA sequence alignment and tree generation

The ARB software package was used to align the 16S rRNA genes from the metagenomic library and to construct a phylogenetic tree. Unlike other sequence alignment software, ARB can take into account the secondary structure of the rRNA in the alignment and provides a comprehensive package of interacting tools

controlled by a single user interface (Ludwig *et al*, 2004). ARB version 06.03.31 and the ssu_jan04_corr_opt_course.arb dataset from the authors' homepage (http://www.arbe-home.de) were used. Imported sequences were initially aligned with the ARB Fast Aligner using the automatically determined most similar reference sequence. The alignment was manually refined using the Secondary Structure Editor. The aligned sequences were inserted into the tree using the ARB-parsimony tool and the tree was optimised using the nearest-neighbour interchange tree modification. The resulting tree was exported from ARB into the Xfig software (Supoj Sutanthavibul), where its appearance was further refined.

2.5.2. Homology modelling

To generate a 3D homology model of Pbpra3229, the primary sequence of the protein was processed through the 3D-JIGSAW Protein Comparative Modelling Server (Bates *et al*, 2001) and the output PDB file was imported into PyMol (DeLano Scientific). The model was aligned to the crystal structures of DiaA and GmhA available from the Protein Databank. Ribbon models were raytraced before the images were taken.

2.6. Flow cytometry analysis

The flow cytometry experiments were performed as previously described with modifications (Keyamura *et al*, 2007). The defined *E. coli* strains were grown overnight at 30°C in LB media supplemented with thymine (50µg/ml). The cultures were diluted 50-fold in LB with either glucose (0.2% w/v) or arabinose (0.01, 0.05 or 0.2% w/v) and incubated at 30°C, with shaking, until an OD_{600} of 0.2. Cephalexin

(10µg/ml) and rifampicin (300µg/ml) were then added, respectively in order to prevent septation and to allow cells to complete the current round of DNA replication without initiating the next round. The cultures were incubated as before for another 4 hours and then the cells were pelleted by centrifugation, washed in buffer (100mM Tris, 10mM $MgSO_4$, pH 7.5), fixed in 70% (v/v) ethanol and stored at 4°C until required. Immediately before use, the cell pellets were washed again with buffer, the DNA stained with SYTOX Green (2µM prepared in buffer) and then the cells were analysed by flow cytometry on a FACScalibur (Becton-Dickinson) using the FITC laser and filters (488nm excitation, 518nm emission) to visualise SYTOX Green. Samples were run through the cytometer under low pressure and the following cytometer settings were used: forward scatter E02, side scatter 350V, FITC laser 480V. The data was analysed using the CellQuest software (Becton-Dickinson), normalised using cells alone and SYTOX without cells. Chromosome number was determined by comparing to a stationary phase culture, where the DNA is not replicating and cells show a peak corresponding to one chromosome per cell.

Chapter 3

P. profundum SS9 DiaA and high pressure adaptation

Among the *P. profundum* SS9 mutants available for study, FL23 had been initially characterised as both pressure-sensitive in liquid medium but was subsequently found to also be cold-sensitive on solid medium (Lauro *et al*, 2008). The transposon insertion was mapped to the *pbpra3229* gene, whose product was annotated as a putative phosphoheptose isomerase (GmhA) but shares higher homology to DiaA, a protein involved in initiation of DNA replication (Section 1.2.2). This led to the hypothesis that *P. profundum* SS9 encodes a DiaA homologue that is essential for growth at high pressure. Further characterisation of the mutant would resolve the true function of the protein and shed light on its role in the ability of *P. profundum* SS9 to adapt to high pressure.

3.1. Physiological characterisation of *P. profundum* FL23

The FL23 mutant was isolated as a pressure-sensitive mutant in liquid medium at 45MPa and 17°C. Further characterisation of the mutant had confirmed the pressure sensitivity at 45MPa but also identified a cold-sensitive phenotype at 0.1MPa and 4°C (Lauro *et al*, 2008). Pressure sensitivity was determined by calculating the ratio of the optical density of early exponential phase cultures of the mutant at 45MPa and 0.1MPa (Lauro *et al*, 2008). In order to expand on these findings the growth on solid and liquid media and the morphology of FL23 were compared to that of the parent strain SS9R at 0.1 and 28MPa. The latter value was chosen because it is the optimal pressure for growth of *P. profundum* SS9.

3.1.1. FL23 has a generalised growth defect and a cold-sensitivity phenotype at atmospheric pressure in liquid culture

The growth of the FL23 mutant and the parent strain SS9R in liquid medium were monitored over a range of pressures and temperatures. At each time point, growth of the sample culture was assessed by measuring the optical density at 600nm. Attempts at assessing the number of colony forming units (CFU) by plating serial dilutions were unsuccessful due to a lack of reproducibility, possibly due to the plating conditions (0.1MPa and 15°C) not corresponding to the optimal growth conditions of *P. profundum* SS9. FL23 has a reduced growth rate at 28MPa and 15°C relative to the parent strain (Figure 3-1 A), however the defect is not as drastic as was observed at 45MPa (Lauro *et al*, 2008). Additionally FL23 displays a similarly reduced growth rate relative to SS9R at 0.1MPa and 15°C (Figure 3-1 B). Lowering the growth temperature to 4°C at 0.1MPa resulted in the most severe growth defect for FL23 (Figure 3-1 C), again consistent with previously published results. Interestingly, lowering the temperature to 4°C at 28MPa did not noticeably affect the growth rate (Figure 3-1 D), suggesting that the stress induced by cold temperature is not as disruptive to the cells at 28MPa as it is at 0.1MPa. Taken together these data show that FL23 has a general growth defect in liquid medium compared to SS9R that is exacerbated at 0.1MPa and 4°C, although the defect seen at 28MPa was not as dramatic as that previously observed at 45MPa (Lauro *et al*, 2008).

3.1.2. FL23 has a generalised growth defect and fails to show high pressure-adaptation on solid agar

The growth defects of certain *P. profundum* SS9 mutants differ depending on

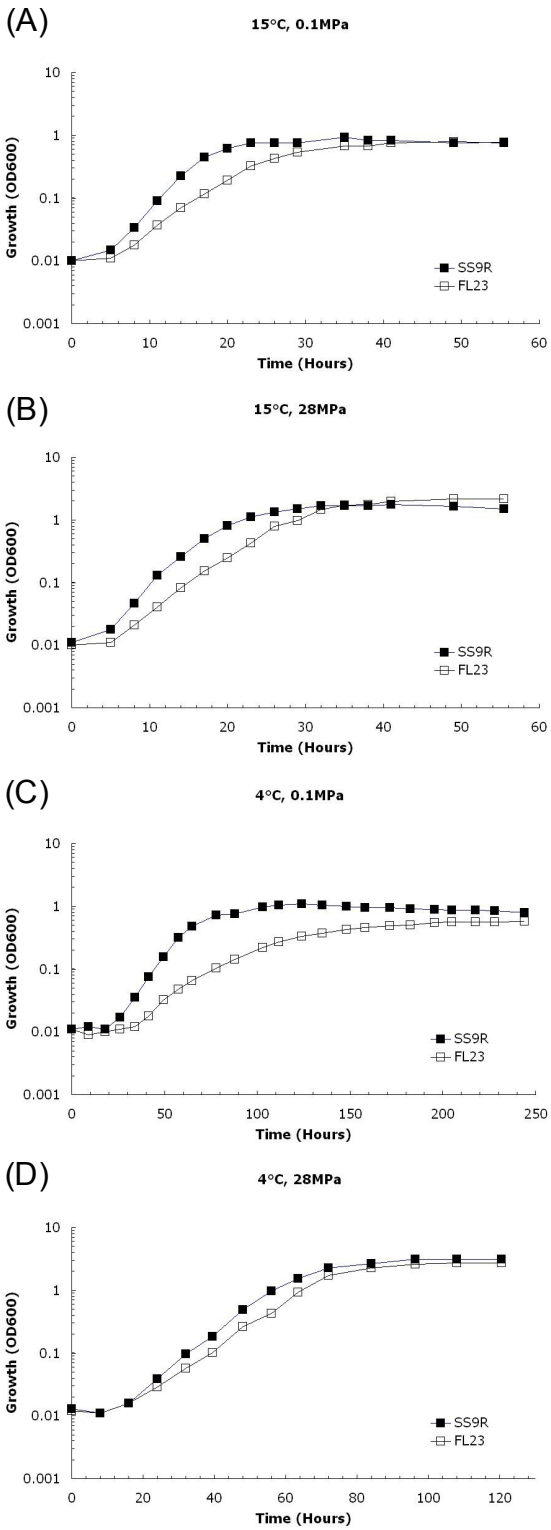


Figure 3-1. Growth of SS9R and FL23 in liquid medium assessed by OD_{600}. Strains were pre-grown at 0.1MPa and 15°C for 48 hours, diluted 1:50 and grown under the same conditions until late exponential phase. They were then diluted to an OD_{600} of 0.01 and grown under the indicated conditions. At each time point growth was assessed by measuring OD_{600}. Experiment was reproduced with similar results.

whether they are grown in liquid or on solid media (Allcock DJ and Ferguson GP, unpublished data). Since changes in cell shape can alter the correlation between cell number and OD measurement of liquid cultures, the growth of FL23 was assessed on solid medium at 0.1MPa over a range of temperatures. Serial dilutions of cultures from SS9R and FL23 were spotted on Marine agar plates and incubated at 0.1MPa and either 15, 9 or 4°C for 96 hours. Under all conditions observed the growth of FL23 was severely inhibited compared to that of SS9R, as the parent strain was consistently able to grow to a lower serial dilution than the mutant (Figure 3-2 A-C). Interestingly after 15 days FL23 was still unable to grow to the same dilution as SS9R (Figure 3-2 D), suggesting that the observed defect is not due to to slower growth in the mutant but to a reduction in colony forming ability. Taken together these data show that the mutation in FL23 is deleterious to growth on agar at atmospheric pressure under all tested temperatures. Since it was possible that the reduced growth could be due to a shift in the temperature range (and therefore of the optimum) towards higher temperature values, the growth of the mutant was also investigated at 20°C, a temperature at which *P. profundum* SS9 grows very poorly. The mutant strain did not show any improvement in growth compared to the parent at 20°C (Figure 3-2 E), suggesting that the mutation in FL23 resulted in a general growth defect that is independent of the temperature at 0.1MPa.

Since the growth defect observed for FL23 on Marine agar was more substantial than the defect seen in Marine broth (compare Figure 3-1 and Figure 3-2), this raised the possibility that the agar composition may be inhibiting the growth of FL23. To test this hypothesis, growth of SS9R and FL23 was assessed on solid medium where agar was substituted for agarose, since agarose is the pure gelling

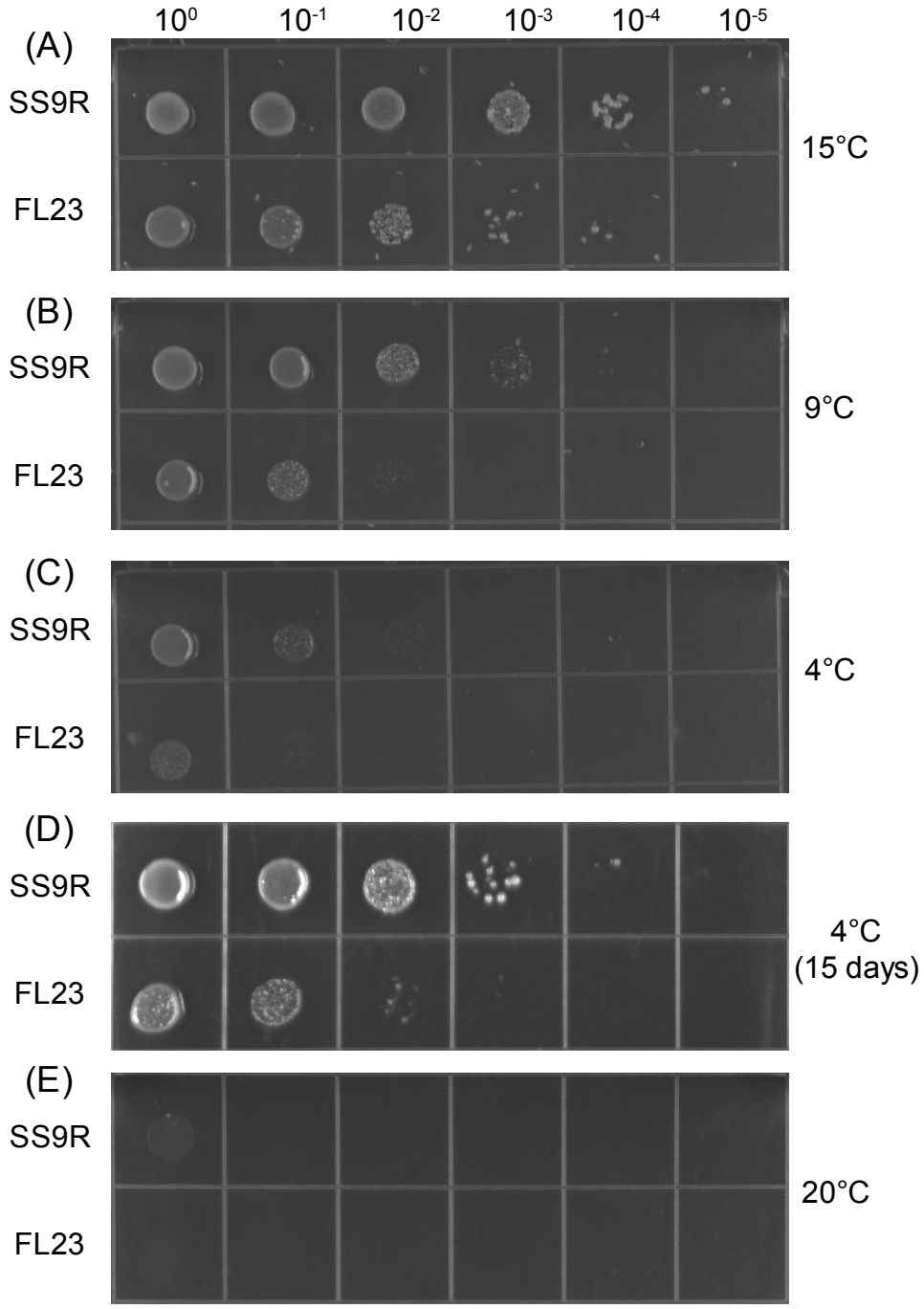


Figure 3-2. Growth of SS9R and FL23 on Marine agar at atmospheric pressure. Liquid cultures were grown to early stationary phase in Marine broth, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted on Marine agar (supplemented with 100mM HEPES and 20mM glucose). Plates were incubated at 0.1MPa at the respective temperatures for 96h unless otherwise indicated. Experiment was repeated twice with similar results.

fraction of agar (Lahaye and Rochas, 1991). Although both strains seemed to grow better on Marine agarose than agar, growth of FL23 was still defective compared to that of SS9R (Figure 3-3), suggesting that the growth defect is due to growth on a solid surface rather than the difference in composition between Marine broth and Marine agar.

Osmotic shock induced by NaCl is known to decrease cell viability and growth in *E. coli* and mutations that affect DNA replication or the cell membrane can affect the sensitivity of bacteria to NaCl (Poirier *et al*, 1998). Difco Marine Broth 2216 (BD), used in making both Marine broth and agar in this study, has an NaCl concentration of 0.33M. To test whether the *pbpra3229* mutation increased the sensitivity of FL23 to NaCl, SS9R and FL23 were grown on Marine agar supplemented with 0.1M NaCl. Increasing the NaCl concentration by 0.1M had no visible effect on SS9R (Figure 3-4 A & B) but this same increase completely inhibited growth of FL23 (Figure 3-4 C & D), suggesting that Pbpra3229 is essential for the ability of *P. profundum* SS9 to tolerate higher salt concentrations.

Characterisation of the pressure sensitivity of FL23 had been studied at 45MPa in liquid medium (Lauro *et al*, 2008). In order to investigate whether FL23 showed a high pressure defect on solid agar, its growth was compared to that of SS9R at 28MPa and 15°C, the optimal growth conditions for *P. profundum* SS9 (DeLong, 1986), using a low gelling temperature agar overlay method (Section 2.1.3). The overlay procedure itself affects the growth of both strains at atmospheric pressure (Figure 3-5 A), possibly due to the increased agar concentration used in this method and because the molten agar sandwich kills a significant proportion of cells due to the heat sensitivity of *P. profundum* SS9. This was compensated for by

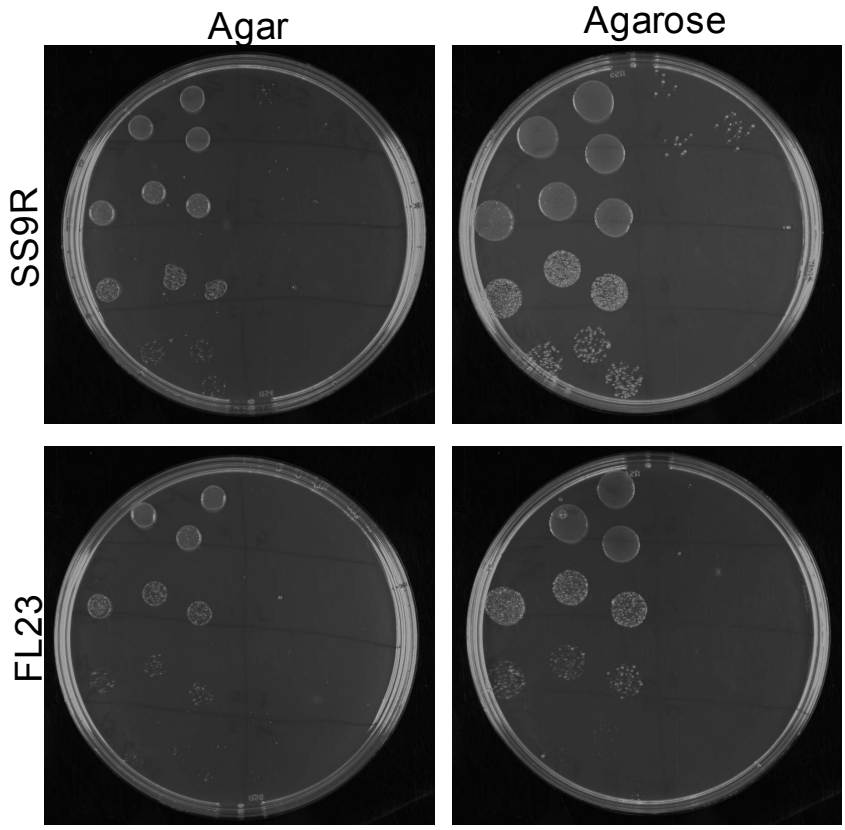


Figure 3-3. Growth of SS9R and FL23 on Marine agar and agarose. Liquid cultures were grown to early stationary phase in Marine broth, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-6}) and spotted in triplicate on Marine agar or Marine agarose (supplemented with 100mM HEPES and 20mM glucose). Plates were incubated at 0.1MPa and 15°C for 96h.

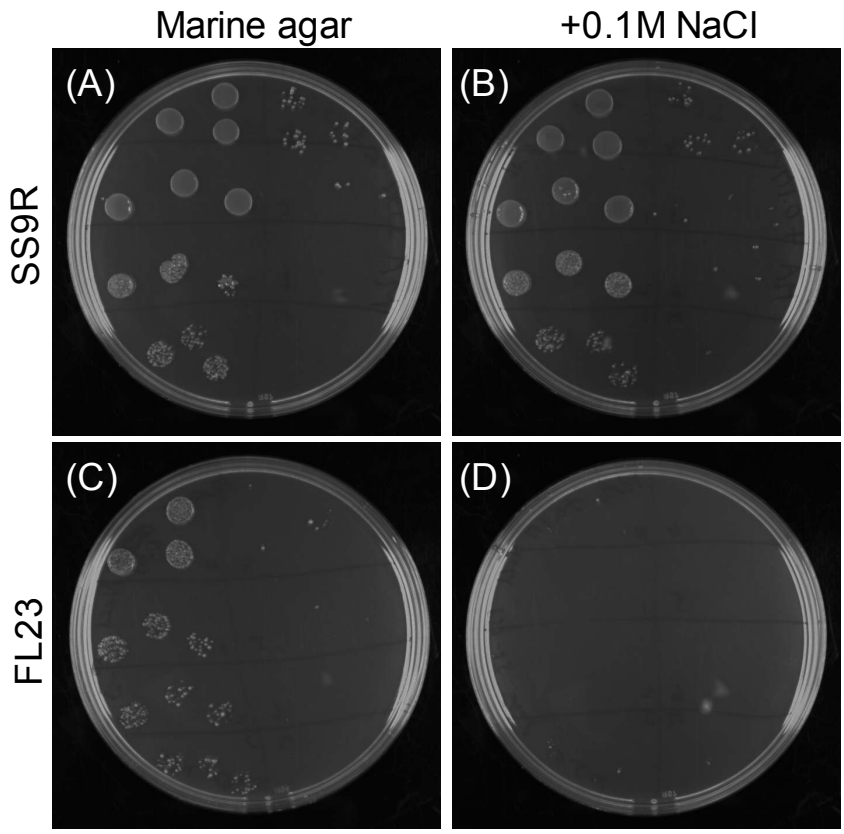


Figure 3-4. Increased sensitivity of FL23 to sodium chloride. Liquid cultures were grown to early stationary phase in Marine broth, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted in triplicate on Marine agar (supplemented with 100mM HEPES and 20mM glucose) with 0.1M added NaCl where indicated. Plates were incubated at 0.1MPa and 15°C for 96h. Experiment was reproduced with similar results.

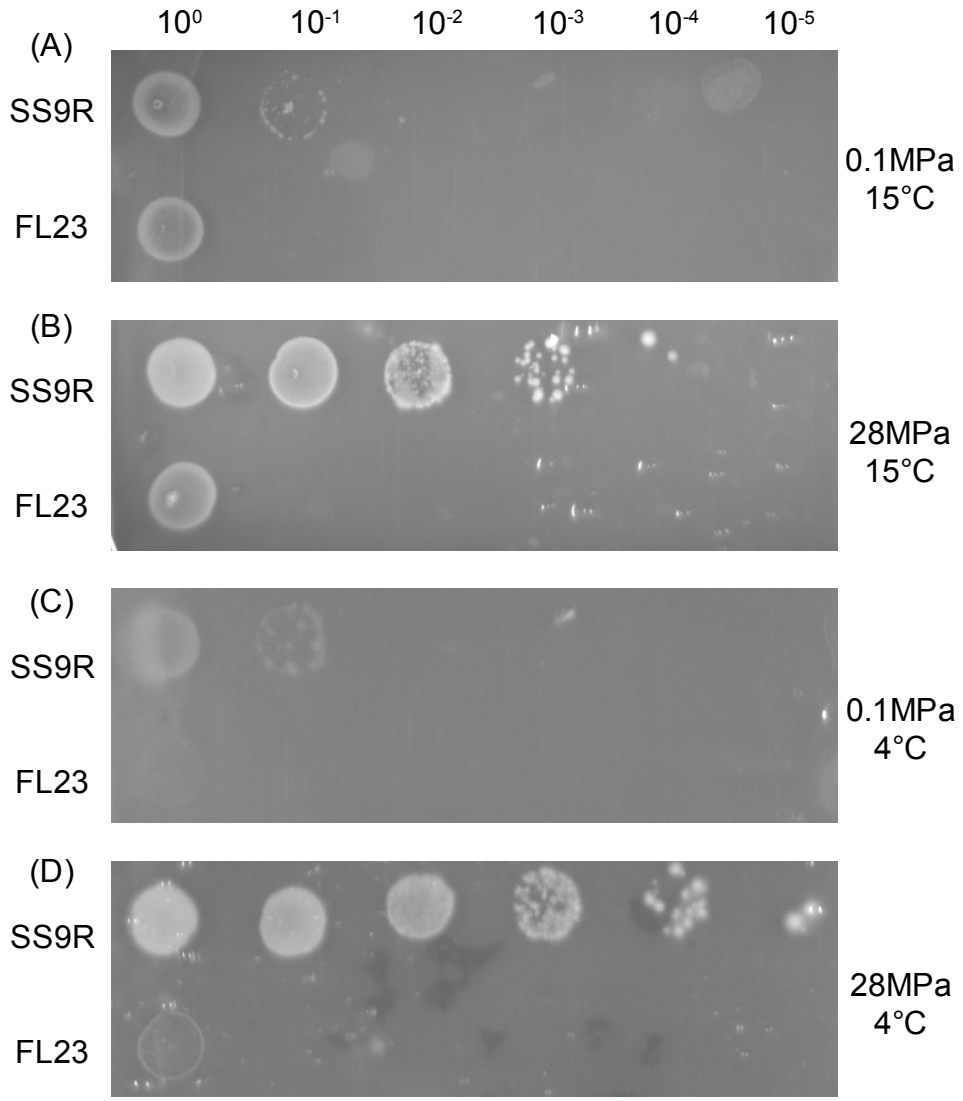


Figure 3-5. Growth of SS9R and FL23 under pressure. Liquid cultures were grown to early stationary phase in Marine broth, diluted to an OD_{600} of 0.8, then serially diluted (10^{-1}-10^{-5}) and spotted on 2.5% LTG Marine agar (supplemented with 100mM HEPES and 20mM glucose). The plates were incubated at 15°C and 0.1MPa for 2 hours, then overlaid with 2.5% LTG Marine agar. Plates were incubated at indicated conditions for 96h (A & B) or 15 days (C & D). Experiment was repeated twice with similar results.

increasing the OD_{600} of the 10^0 dilution from 0.2 to 0.8. Under these conditions SS9R stops growing at the 10^{-1} dilution at 0.1MPa and 15°C but can form colonies on the 10^{-4} dilution at 28MPa (Figure 3-5 A & B, respectively), demonstrating that this method provides a reliable way to compare atmospheric and high pressure growth in *P. profundum* SS9. As expected SS9R grew marginally better than FL23 at 0.1MPa and 15°C (Figure 3-5 A). The gap between the growth of SS9R and FL23 substantially widened at 28MPa and 15°C, since SS9R was able to grow to the 10^{-4} dilution whereas FL23 only grew on 10^0 (Figure 3-5 B). Since FL23 only grew on the undiluted spots at both pressures and was not able to improve growth at high pressure, this demonstrates that FL23has lost the ability to adapt to pressure on solid agar at 15°C. This was further tested by repeating the overlay experiment and incubating the plates at 0.1 and 28MPa at 4°C for a longer time (15 days). Again the mutant strain only grew on the undiluted spot at both pressures, however growth was improved at 28MPa compared to 0.1MPa (Figure 3-5 C & D). This suggested that low temperature can partially rescue the pressure adaptation block in FL23, although the strain is still unable to fully adapt when compared to SS9R. Taken together these data provide further evidence that FL23 has lost the ability to adapt to pressure on solid medium and suggest that the loss of pressure adaptation is mostly independent of temperature.

3.1.3. FL23 has abnormal cellular morphologies at high pressure

E. coli cells are rod-shaped at atmospheric pressure and filament at high pressure (Zobell and Cobet, 1962). In contrast, *P. profundum* SS9 follows an opposite trend and previous studies have shown that *P. profundum* SS9 is rod-shaped

at 28MPa and filaments at 0.1MPa (Bidle and Bartlett, 1999). In order to determine whether the growth defect of FL23 is due to impaired cell division, the cell morphology of the mutant cells was examined using phase contrast microscopy and compared to that of the parent strain. As expected, SS9R formed a small number of filaments at 0.1MPa and 15°C but all cells were rod-shaped at 28MPa 15°C (Figure 3-6 A & B respectively). In contrast, FL23 formed a mixture of rods, short filaments and chained cells at both pressures (Figure 3-6 C & D). At 0.1MPa the mutant cells also formed aberrant morphologies and bulbous cell ends that are not observed with the parent strain under either condition (Figure 3-6 C). This suggests that the inactivation of *pbpra3229* impairs cell division and this could be the reason behind the growth defect exhibited by FL23 in liquid media. The filaments and abnormal cellular morphologies formed by FL23 could account for the decreased colony forming ability of this mutant and the presence of these morphologies at atmospheric and high pressure provides an explanation for the loss of pressure adaptation seen on solid medium. The OD_{600} measurements seen in the growth curves (Figure 3-1) may also be affected by the aberrant morphologies.

In order to examine further the morphology of the mutant cells and observe if more subtle abnormalities can be seen at the ultrastructure level, SS9R and FL23 cells were fixed then sent to Euan James (University of Dundee) to be examined by transmission electron microscopy. The morphology of SS9R cells was mostly uniform at 15°C at 0.1 and 28MPa (Figure 3-7 A & B respectively). FL23 cells showed some elongated cells at 28MPa and 15°C (Figure 3-7 D), in addition to cells joined at their septa (Figure 3-7 F). At atmospheric pressure the mutant cells displayed strikingly abnormal morphologies, including lemon-shaped poles and

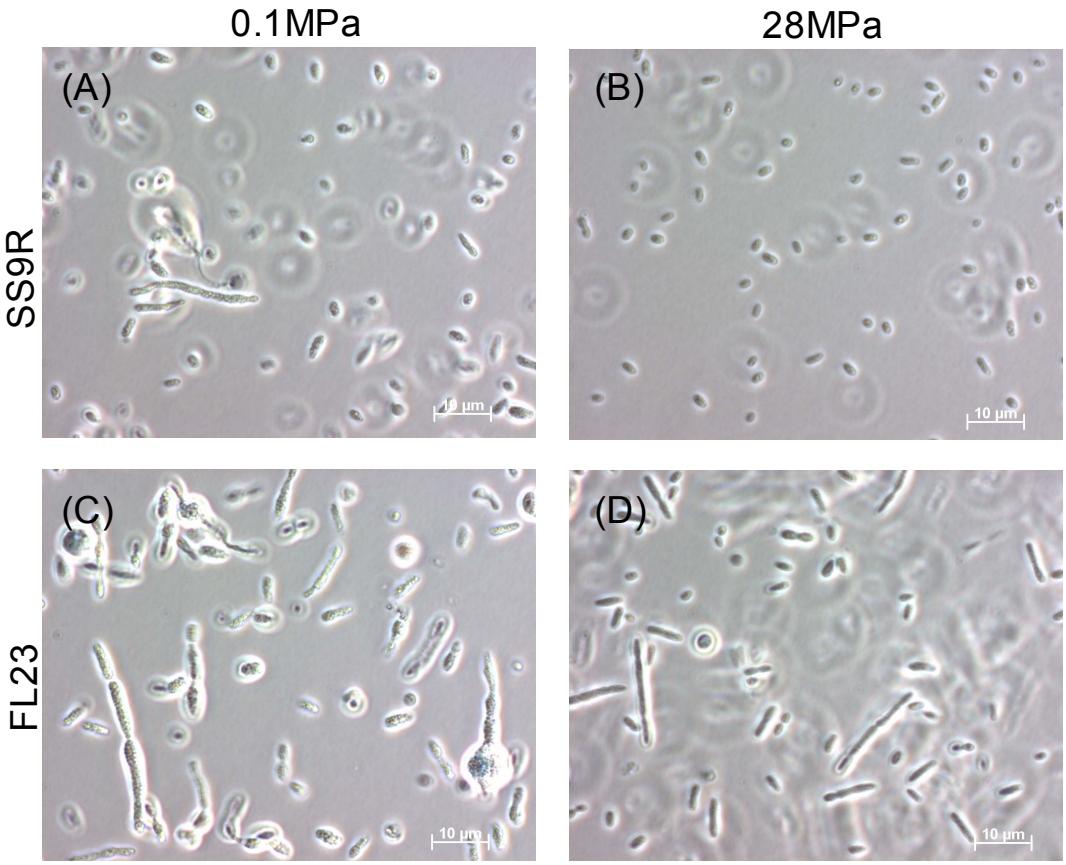


Figure 3-6. Cell morphology of SS9R and FL23 by phase contrast microscopy. Liquid cultures were grown to early stationary phase in Marine broth at the indicated pressure, and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. Scale bars represent 10µm. Experiment was reproduced with similar results.

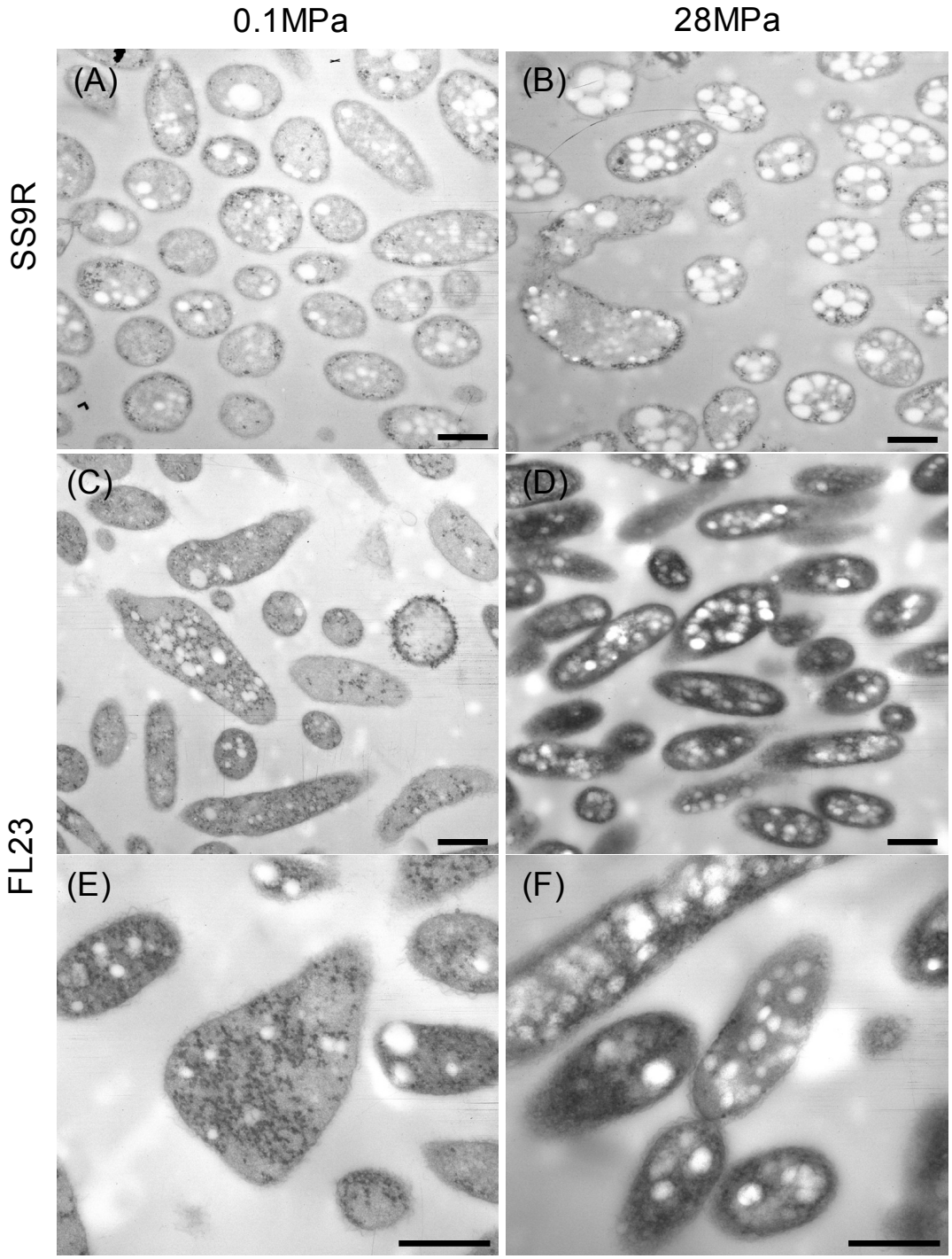


Figure 3-7. Cell morphology of SS9R and FL23 by TEM. Liquid cultures were grown to early stationary phase in Marine broth at the indicated pressure, fixed in Karnovsky fixative, dehydrated then sectioned and imaged on a transmission electron microscope. Images were taken at 10,000x (A-D) or 20,000x magnification (E-F). Scale bars represent 1µm.

triangular shapes (Figure 3-7 C & E). This confirms that FL23 forms a mixture of rods and cells with aberrant morphologies compared to the parent strain SS9R.

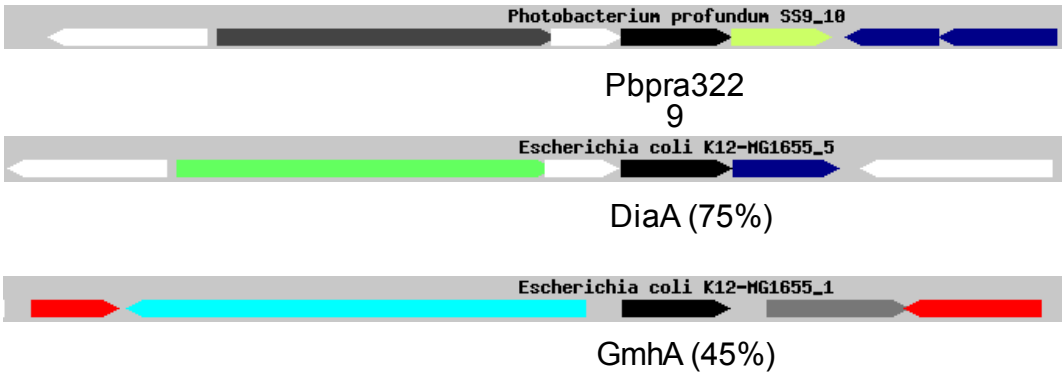
3.2. Function of *P. profundum* SS9 Pbpra3229

As previously discussed (Section 1.2.2), Pbpra3229 was originally annotated as a phosphoheptose isomerase (GmhA) when the *P. profundum* SS9 genome was sequenced (Vezzi *et al*, 2005), due to the presence of a SIS (sugar isomerase) domain usually found in phosphosugar-binding proteins (Bateman, 1999). However Pbpra3229 has 75% amino acid identity to *E. coli* DiaA and only 45% identity to *E. coli* GmhA. Studies were carried out with FL23 to identify defects in either LPS biosynthesis or DNA replication and therefore conclude if Pbpra3229 was the *P. profundum* SS9 homologue of GmhA or of DiaA.

3.2.1. Sequence and structural homology between Pbpra3229, DiaA and GmhA

Pbpra3229 shares 45% amino acid identity with GmhA, the *E. coli* phosphoheptose isomerase, but shares 75% identity with *E. coli* DiaA. Since both values are relatively high, another method was investigated to determine the most likely homologue of *pbpra3229*. The J. Craig Venter Institute Comprehensive Microbial Resources project provided a BLAST algorithm that compares the entire genomic region around the gene of interest and looks for homologous regions in other organisms. Using this Genome Region Comparison algorithm, the region around Pbpra3229 was much more similar to that of DiaA rather than GmhA (Figure 3-8 A). Both Pbpra3229 and DiaA were the third in a putative operon of four genes,

(A)



(B)

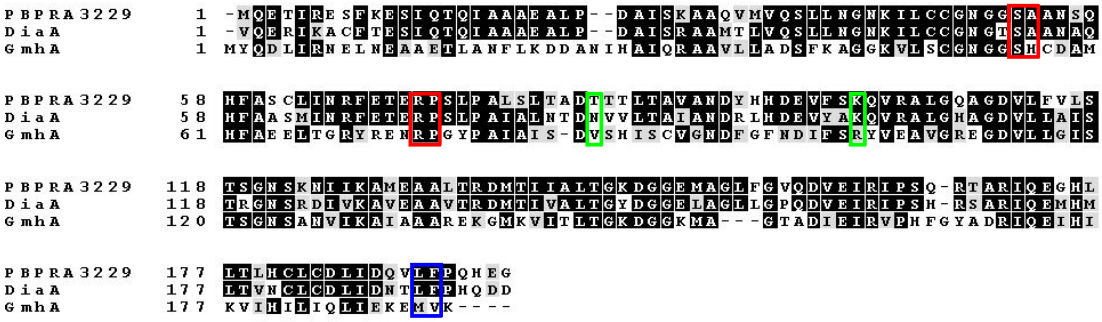


Figure 3-8. Sequence alignment and genome region comparison of *pbpra3229* and its gene product. A: The *pbpra3229* gene region was compared to that of *E. coli diaA* and *gmhA* using the J. Craig Venter Institute Genome Region Comparison. The percentages are protein identities with respect to Pbpra3229. B: Sequence alignment of Pbpra3229, DiaA and GmhA using ClustalW. Black residues are identical and grey residues have similar chemical properties. Boxed residues are those with known function in DiaA: blue interact with DnaA, green are involved in tetramer formation and red are essential for DiaA function.

with the respective genes in *E. coli* and *P. profundum* SS9 being of comparative lengths, whereas both regions were very dissimilar to that of *E. coli* GmhA. The genome of *P. profundum* SS9 contains another gene, *pbpra2913*, whose product is also annotated as a phosphoheptose isomerase and shares 75% amino acid identity with *E. coli* GmhA. Taken together these data suggested that Pbpra2913 is a *P. profundum* SS9 GmhA homologue while Pbpra3229 is likely to be a *P. profundum* SS9 DiaA homologue.

In order to identify conserved domains between *P. profundum* SS9 Pbpra3229 and *E. coli* DiaA and GmhA the amino acid sequences of the three proteins were aligned with ClustalW. The alignment showed Pbpra3229 and DiaA shared a large number of conserved domains whereas GmhA did not share as many conserved domains with either Pbpra3229 or DiaA (Figure 3-8 B). Since the importance of some amino acid residues in the biochemical functions of DiaA and GmhA had previously been identified (Keyamura *et al*, 2007), these were compared to their corresponding residues in Pbpra3229. Four residues that are essential for DiaA function were conserved in Pbpra3229, although interestingly three of these were also conserved in GmhA. One of two residues involved in tetramer formation in DiaA was conserved in Pbpra3229 whereas the other was not conserved among any of the proteins. Most importantly, the two DiaA residues known to be involved in binding DnaA were conserved in Pbpra3229 but not GmhA. This provides further evidence that Pbpra3229 is more likely to be a DiaA homologue.

GmhA and DiaA had been shown to have very similar crystal structures even though the proteins have unrelated functions (Seetharaman *et al*, 2006; Keyamura *et al*, 2007). As previously mentioned (section 1.1.1) extremophiles can have structural

modifications that allow them to cope with the extremes that characterise their habitat. To get an indication of whether Pbpra3229 had obvious structural modifications, a homology model of the protein in tetrameric form was generated from the *E. coli* DiaA structure using the 3D-JIGSAW Comparative Modelling Server (*Bates et al*, 2001) and compared to the published crystal structures of DiaA and GmhA. No significant differences could be observed between the modelled structure of the Pbpra3229 tetramer and the resolved crystal structure of DiaA (Figure 3-9 A & B respectively). Additionally, some differences could be seen between the two proteins and GmhA (Figure 3-9 C), although the general structure of the tetramer was highly similar in all three cases.

3.2.2. Pbpra3229 is probably not a phosphoheptose isomerase

GmhA is well-studied and its enzymatic function characterised. *E. coli* X711, a *gmhA* null mutant, cannot synthesise the inner core and as a result the LPS molecules are truncated (Section 1.3). This phenotype can be readily visualised using SDS-PAGE by sodium-m-periodate and silver staining (Figure 1-8 ; Brooke and Valvano, 1996). This truncated LPS makes the outer membrane less stable and consequently the mutant suffers from increased sensitivity to antibiotics (Tamaki *et al*, 1971).

Although the higher homology to *E. coli* DiaA suggested that Pbpra3229 was involved in initiation of DNA replication, the possibility that it was a homologue of GmhA could not be ruled out. To determine whether Pbpra3229 also functioned as a phosphoheptose isomerase, the LPS of SS9R and FL23 was extracted from cultures grown at at 0.1MPa and 15°C and analysed by SDS-PAGE. The gel was stained with

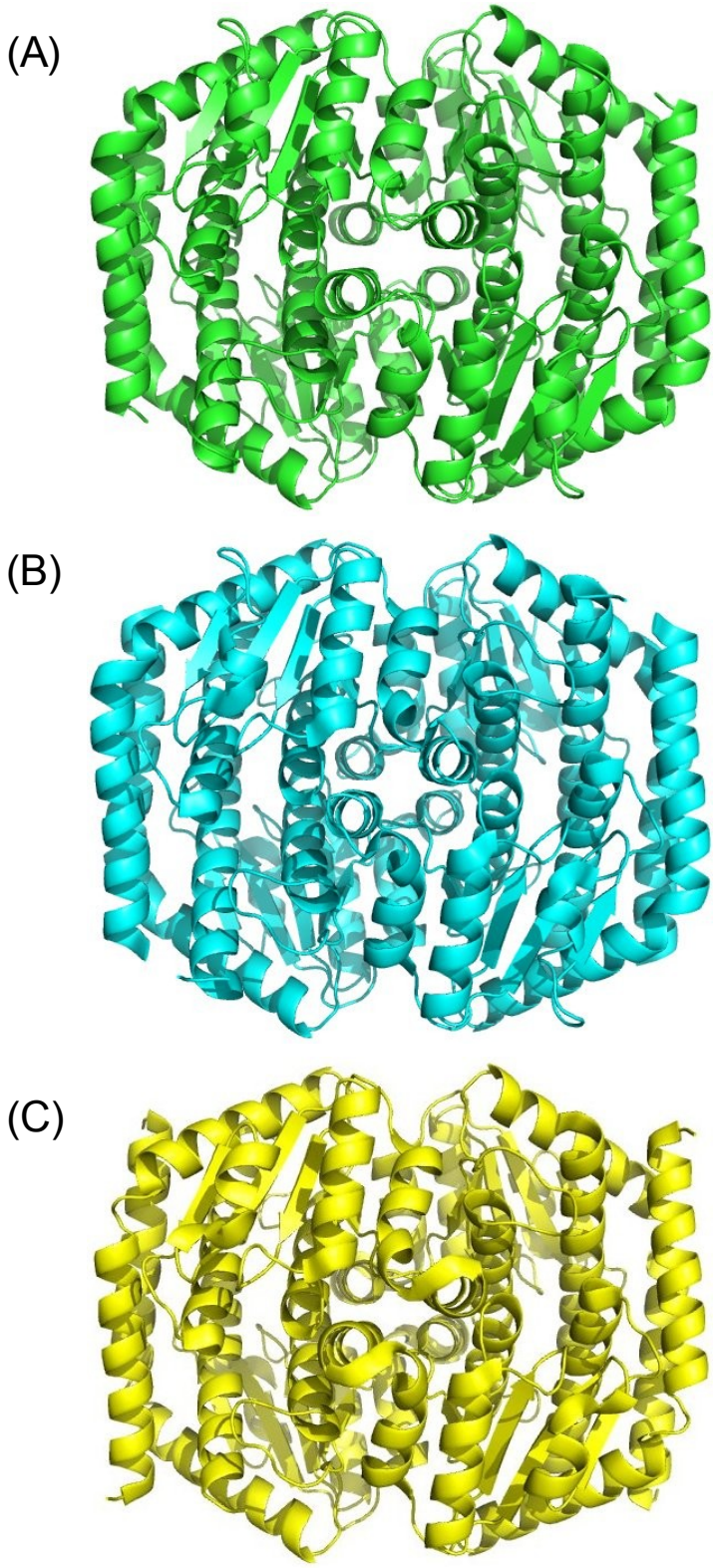


Figure 3-9. Crystal structures of *E. coli* DiaA and GmhA and homology model of *P. profundum* Pbpra3229. The crystal structures were retrieved from the RCSB Protein Data Bank. The homology model was constructed using the 3D-JIGSAW Comparative Modelling Server. Images were taken with PyMol. A: Pbpra3229; B: DiaA; C: GmhA.

sodium-m-periodate and silver nitrate using the previously published methodology (Brooke and Valvano, 1996). The LPS from X705 and X711 (the *E. coli* parent and *gmhA* deletion mutant, respectively) were used as controls to confirm that the mutant LPS phenotype could be reproduced. Using this technique, the LPS profiles of SS9R and FL23 were identical and no band shift corresponding to the truncated LPS could be observed, whereas X711 displayed the characteristic truncated LPS (Figure 3-10A). This shows that FL23 does not lack the inner core of the LPS and suggests that disruption of *pbpra3229* does not impair biosynthesis of *glycero-manno*-heptose at 0.1MPa and 15°C. *E. coli* KH5402-1 and NA141 (the *E. coli* parent and *diaA* mutant, respectively) were also used and as expected did not show LPS alterations.

In order to further confirm that Pbpra3229 is not involved in biosynthesis of the inner core of the LPS, cultures of SS9R and FL23 were grown under different combinations of temperature and pressure. In addition to sodium-m-periodate staining, the LPS was also stained with Alcian blue, a cationic dye that binds acidic residues and can be used to visualise LPS modifications in *P. profundum* SS9 (Corzo *et al*, 1991). No noticeable difference in LPS profile could be seen between SS9R and FL23 under the optimal growth conditions or in response to low temperature, atmospheric pressure or both, using either sodium-m-periodate or Alcian blue in combination with silver staining (Figure 3-10 B & C, respectively). Therefore FL23 does not display the same LPS alteration as X711 on SDS-PAGE, suggesting that Pbpra3229 and GmhA do not share identical functions. Taken together these data show that *pbpra3229* is unlikely to encode a phosphoheptose isomerase.

Although there were no noticeable differences between SS9R and FL23, both strains had alterations in the banding patterns over different pressure and temperature

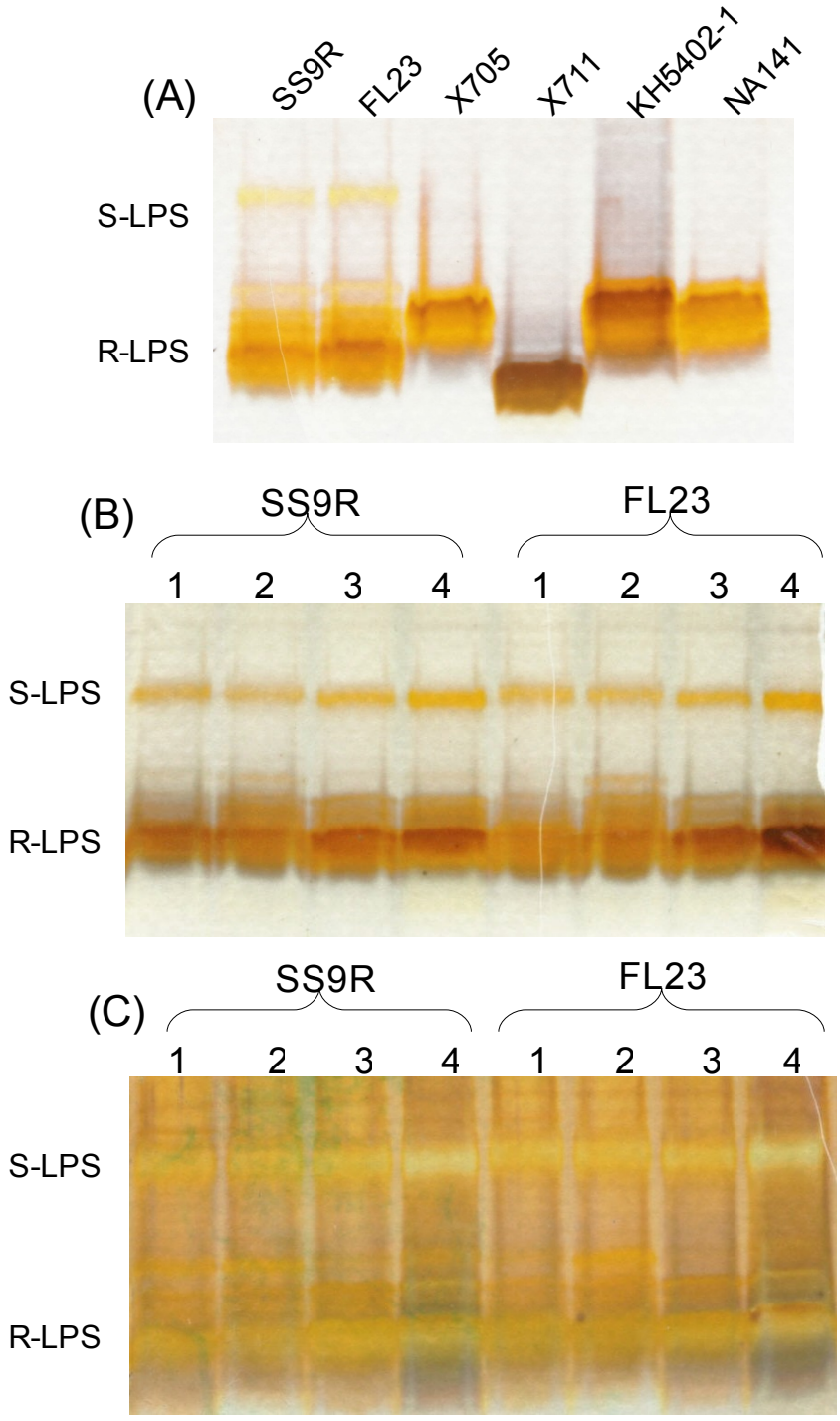


Figure 3-10. LPS profiles of the *pbpra3229*, *gmhA* and *diaA* mutants. The *E. coli* parent strains (X705 and KH5402-1) and the *gmhA* (X705) and *diaA* (NA141) mutants were grown at 37°C. *P. profundum* strains were grown at either at 28MPa and 15°C (1), or 0.1MPa and 15°C (2), 9°C (3) or 4°C (4). LPS was extracted using standard SDS lysis and analysed by SDS-PAGE. The gel was oxidised with sodium-m-periodate (0.7% in fixative) and silver stained (A & B) or stained with alcian blue and silver (C). The smooth (S-LPS) and rough (R-LPS) are denoted. Experiment was reproduced with similar results.

growth conditions (Figure 3-10 B & C). Even though the uppermost (smooth LPS) and lowermost (rough LPS) bands were invariant, the intensity of the intermediate bands seemed to vary, and some were entirely absent under certain conditions, especially when visualised using sodium-m-periodate and silver staining (Figure 3-10 B). This suggested that SS9R can modulate its LPS in response to environmental conditions and that the LPS could play a role in high pressure and cold temperature adaptation in *P. profundum* SS9 (Allcock DJ and Ferguson GP, unpublished data).

3.2.3. Cloning of the pbpra3229 and diaA genes

The broad-host range vector pFL190 (Lauro *et al*, 2005) was chosen for cloning because of its ability to replicate in both *E. coli* and *P. profundum* SS9 and because genes can be cloned under the control of an arabinose-inducible promoter. This was of particular importance since *E. coli diaA* and *pbpra3229* are the third genes in their respective putative operons and may not have a promoter directly upstream. The *E. coli diaA* and *P. profundum* SS9 *pbpra3229* genes were amplified by PCR and cloned into pFL190 (Section 2.3.6). The pEC*diaA* and p*pbpra3229* plasmids were thus generated, carrying the *E. coli diaA* and *P. profundum* SS9 *pbpra3229* genes respectively.

3.2.4. Pbpra3229 can complement the diaA mutation in E. coli

The alignment of the Pbpra3229 and *E. coli* DiaA sequences, the homology model of Pbpra3229 and the presence of the two genes within very similar operons all suggested that Pbpra3229 was a homologue of DiaA. DiaA is a positive regulator of DNA replication that binds DnaA and promotes initiation of replication at the

origin (Section 1.4.2). Evidence that Pbpra3229 and DiaA are homologues was collected by assessing the ability of Pbpra3229 to substitute for *E. coli* DiaA. The *E. coli diaA* mutant NA141 had previously been shown to have an identical doubling time to its parent strain as measured by OD_{600} even though timely initiation of DNA replication was disrupted, as seen by flow cytometry (Ishida *et al*, 2004). Microscopic examination of NA141 cells revealed no filaments or abnormal morphologies (Figure 3-11 A & B), in striking contrast to *P. profundum* FL23 (Figure 3-6). Since the growth defect in FL23 was more exacerbated on solid medium than in liquid medium (compare Figure 3-1 and Figure 3-2), the growth of NA141 was assessed on LB agar. The colony forming ability of the *E. coli* mutant was also unaffected compared to the parent strain at both 37 and 15°C (Figure 3-11 C & D respectively) and correlated with the unchanged doubling time and cell morphologies. This shows that, unlike *P. profundum* FL23, the *E. coli diaA* mutant has no growth defect or aberrant morphologies despite having a disruption in timely initiation of DNA replication.

Since the *E. coli diaA* mutant had no visible growth phenotype under normal conditions another background was used that produces a mutated DnaA protein. The cold-sensitive *dnaAcos* mutation leads to overinitiation of replication in *E. coli* at 30°C and results in cell division arrest, filamentation and death (Kellenberger-Gujer *et al*, 1978). DiaA was initially identified as an intergenic suppressor of cold sensitivity in the *dnaAcos* mutant (Ishida *et al*, 2004). Disrupting the *diaA* gene in the *dnaAcos* background suppresses cold sensitivity and restores growth at 30°C. Expressing the wild-type *diaA* gene on a plasmid restores cold-sensitivity in the *E. coli dnaAcos diaA* double mutant NA026 (Ishida *et al*, 2004). This was taken

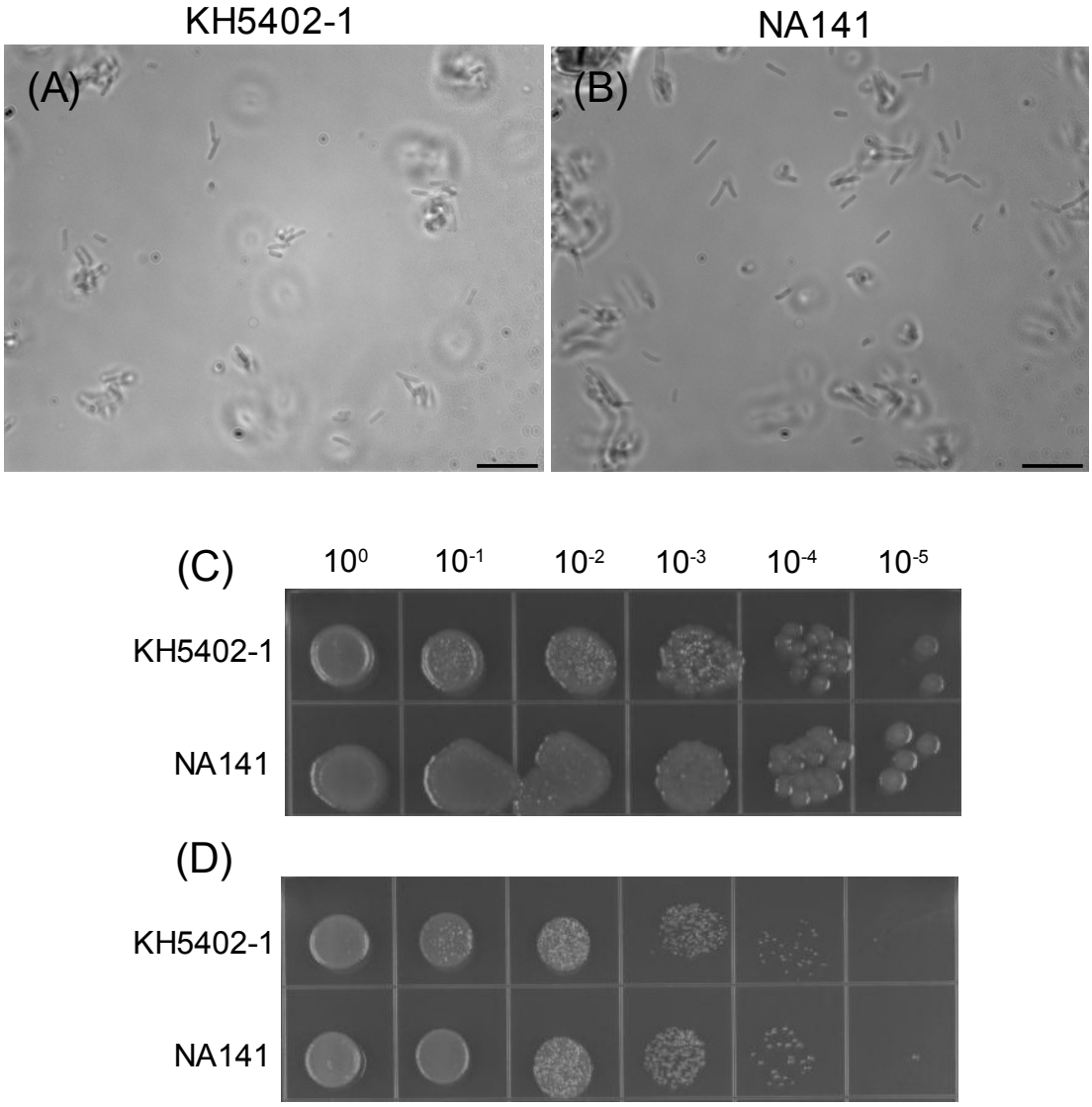


Figure 3-11. Growth of the *E. coli diaA* mutant. A & B: The *E. coli* parent (KH5402-1) and mutant (NA141) were grown overnight in LB broth supplemented with thymine and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. C & D: Liquid cultures were grown overnight in LB broth supplemented with thymine, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted on LB agar supplemented with thymine. Plates were incubated at 37°C overnight (C) or 15°C for 2 days (D). Experiment was reproduced with similar results.

advantage of to test if Pbpra3229 could also restore the cold-sensitive phenotype in NA026.

The pFL190, pEC*diaA* and p*pbpra3229* plasmids were transformed into NA026; pFL190 was also transformed into the parent strain KH5402-1 and the *dnaAcos* mutant NA001. The cold-sensitive phenotype of the *dnaAcos* mutation and its suppression by inactivating *diaA* were reproduced at 30°C (Figure 3-12 A) while all strains grew as well as the parent at 42°C (Figure 3-12 B). Expressing wild-type *E. coli diaA* from pEC*diaA* partially complemented the mutation since the plasmid restored cold-sensitivity, although to a lesser degree than observed with NA001 pFL190 (Figure 3-12 A). However p*pbpra3229* was able to complement the mutation to the same extent as pEC*diaA* since NA026 with either pEC*diaA* or p*pbpra3229* grew to the same dilution. This provided evidence that Pbpra3229 can functionally substitute for *E. coli* DiaA *in vivo*.

3.3.5. Pbpra3229 can restore timely initiation of DNA replication in the E. coli diaA mutant

The ability of *pbpra3229* to complement the *E. coli diaA* mutation in the *dnaAcos* background indicated that Pbpra3229 was likely involved in initiation of DNA replication. Flow cytometry is an established technique used to identify defects in initiation of replication. Cells are treated with rifampicin and cephalexin while in mid-exponential phase to allow them to complete their current cycle of replication but they cannot initiate the subsequent cycle and cannot complete septation and cell division (Skarstad *et al*, 1995). The cells are then fixed, stained with a DNA dye and run through a flow cytometer, which allows to cells to be separated depending on

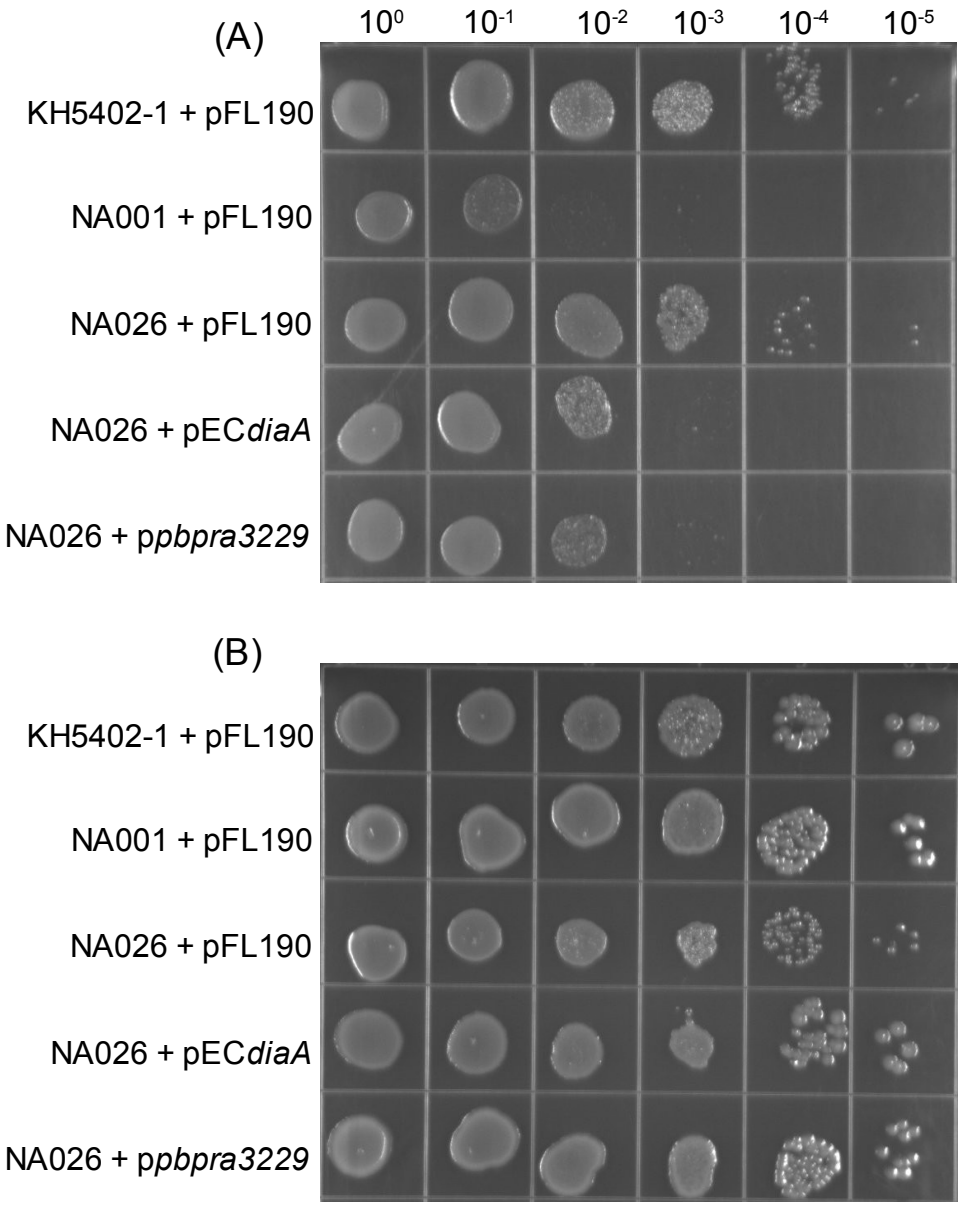


Figure 3-12. Expression of Pbpra3229 in the *E. coli dnaAcos diaA* double mutant. The *E. coli* parent (KH5402-1), *dnaAcos* mutant (NA001) and *dnaAcos diaA* double mutant (NA026) were grown overnight in LB broth supplemented with thymine, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted on LB agar supplemented with thymine, streptomycin (100µg/ml) and arabinose (0.01% w/v). Plates were incubated at 30°C (A) or 42°C (B) overnight. Experiment was repeated twice with similar results.

their DNA content and therefore their number of chromosomes. *E. coli* initiation of DNA replication is synchronous and this results in the formation of cell populations with only even numbers of chromosomes (Figure 3-13 A). Mutants that are defective in timely initiation of DNA replication, such as *the E. coli diaA* mutant, have an asynchronous DNA replication phenotype, resulting in an odd number of chromosomes in some of the cells (Figure 3-13 B; Ishida *et al*, 2004).

As a functional homologue of DiaA, Pbpra3229 should be able to complement the DNA replication defect of the *E. coli diaA* mutant. To investigate this, pFL190, pEC*diaA* and p*pbpra3229* were transformed into NA141 and DNA replication in the three constructs was analysed by flow cytometry and compared to that of the parent strain KH5402-1 carrying pFL190. As previously shown (Ishida *et al*, 2004) the parent strain had discreet peaks corresponding to even numbers of chromosomes, as shown by the fluorescence intensity of the second peak (4 chromosomes) being double that of the first (2 chromosomes) and that of the third (8 chromosomes) being approximately four times that (Figure 3-14 A). Additionally the majority of the cells had 4 chromosomes, again as previously shown (Keyamura *et al*, 2007). The *diaA* mutant carrying the empty vector had multiple peaks corresponding to any number of chromosome, indicating defective initiation of DNA replication (Figure 3-14 B). Supplying wild-type *E. coli* DiaA in the mutant complements the phenotype and restores timely initiation of DNA replication, as seen by the disappearance of the intermediate peaks and the return of the majority of the cells into the 4 chromosomes peak (Figure 3-14 C). Introducing wild-type Pbpra3229 in the mutant complements the phenotype in an almost identical way to *E. coli* DiaA by also eliminating the peaks associated with the mutation and returning

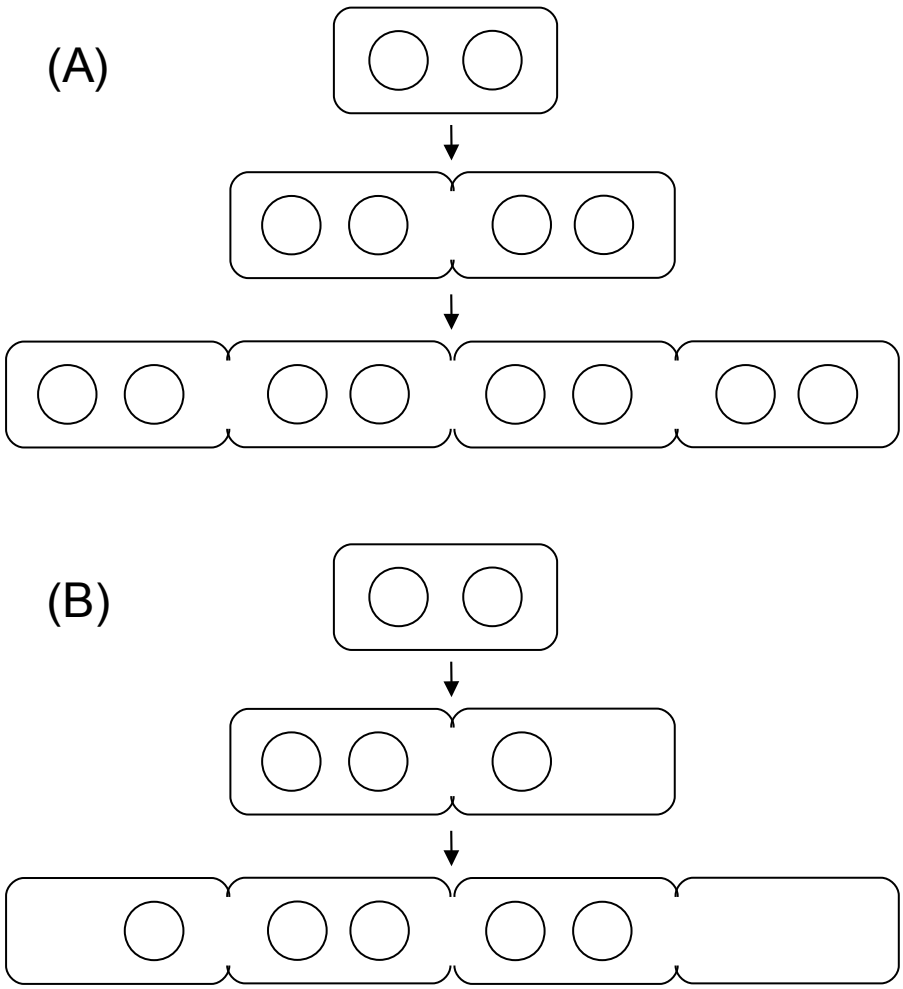


Figure 3-13. Models for growth after treatment for FACS. In the event of timely initiation of DNA replication the cells will always have an even number of chromosomes (A). If timely initiation is disrupted the loss of synchrony leads to a mixture of cells with both even and odd numbers of chromosomes.

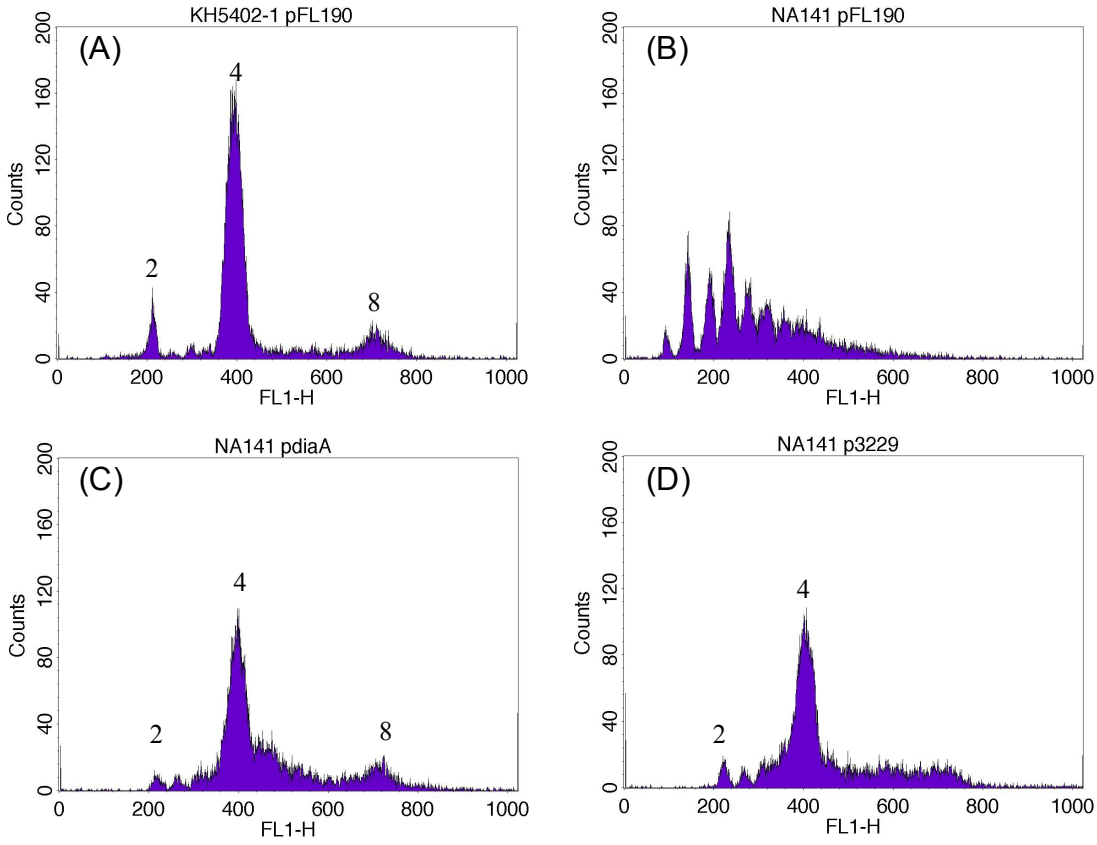


Figure 3-14. Complementation of the initiation of DNA replication defect in the *E. coli diaA* mutant by Pbpra3229. The *E. coli* parent (KH5402-1) and *diaA* mutant (NA141) were grown at 30°C in LB until mid-exponential phase, treated with rifampicin and cephalexin, fixed in 70% ethanol then the DNA was stained with Sytox Green and analysed by flow cytometry. FL1-H is the measured fluorescence intensity using the FITC filter. Chromosome number is indicated above the peaks. Experiment was reproduced with similar results.

most of the cells into the 4 chromosomes peak (Figure 3-14 D). This shows that Pbpra3229 is the *P. profundum* SS9 homologue of DiaA. Subsequently *P. profundum* SS9 DiaA will be referred to as PpDiaA whereas *E. coli* DiaA will be referred to as EcDiaA.

Flow cytometry has also been successfully used in *Vibrio cholerae* to analyse DNA content in initiation-impaired mutants *(*Duigou *et al*, 2006). Since *V. cholerae* is a close relative of *P. profundum* SS9 and also has two chromosomes, attempts were made to adapt the method to work in SS9R. For optimal results cells need to be of uniform shape and size. Several technical issues arose with growing and treating the *P. profundum* SS9 cells at high pressure since the cultures had to remain at atmospheric pressure for significant durations while being treated and fixed. Additionally SS9R, the *P. profundum* SS9 lab strain from which all other mutants are derived, is rifampicin-resistant and can still divide even after treatment. Due to these factors the cells could not be treated properly and attempts at visualising single peaks corresponding to discreet genome equivalents were not consistently reproducible in SS9R.

3.3.6. E. coli DiaA can complement the pressure defect of P. profundum FL23

In order to confirm that the growth defect observed in FL23 is due to the inactivation of *pbpra3229* and not to downstream polar effects of the mini-Tn*10* insertion, the mutant phenotype must be complemented with wild-type *pbpra3229*. Additionally, complementation of the phenotype with *E. coli diaA* would prove that EcDiaA is functional in *P. profundum* SS9. To this end, pFL190, p*pbpra3229* and

pEC*diaA* were conjugated into FL23 using triparental mating while pFL190 was conjugated into SS9R for use as a control. The four constructs were then grown and their colony forming ability was assessed as previously (Section 3.1.2). As expected SS9R pFL190 grew to the 10^{-5} dilution whereas FL23 pFL190 only grew to 10^{-3} (Figure 3-15 A); this confirmed that the introduction of pFL190 had no effect on the growth of the *P. profundum* SS9 strains. Introducing wild-type PpDiaA into FL23 restored the wild-type phenotype, demonstrating that the mutant phenotype is solely due to the disruption of *pbpra3229* and not to polar effects exerted by the mini-transposon. EcDiaA was also able to rescue the defective phenotype of FL23 on solid medium; this shows that EcDiaA can function in *P. profundum* SS9 and can substitute for PpDiaA.

The pressure-sensitive phenotype observed with FL23 raised the possibility that PpDiaA is a high pressure-adapted protein and therefore a high pressure-adapted homologue of EcDiaA. If this were the case, PpDiaA would be able to complement the mutant phenotype of FL23 at both atmospheric and high pressure whereas EcDiaA would only be expected to do so at atmospheric pressure. To test this hypothesis, the growth of the complementation constructs was assessed using the agar overlay method. In early experiments involving pFL190 and its derivatives the addition of streptomycin (used to maintain pFL190, which encodes resistance to the antibiotic) to the low gelling temperature agar plates resulted in much poorer growth and reproducibility. For these reasons streptomycin was omitted from the agar and this did not affect the observed phenotypes at atmospheric pressure, as the mutant's reduced colony forming ability was complemented by both *E. coli* and *P. profundum diaA* (Figure 3-15 B). At 28MPa and 15°C, FL23 pFL190 displayed the

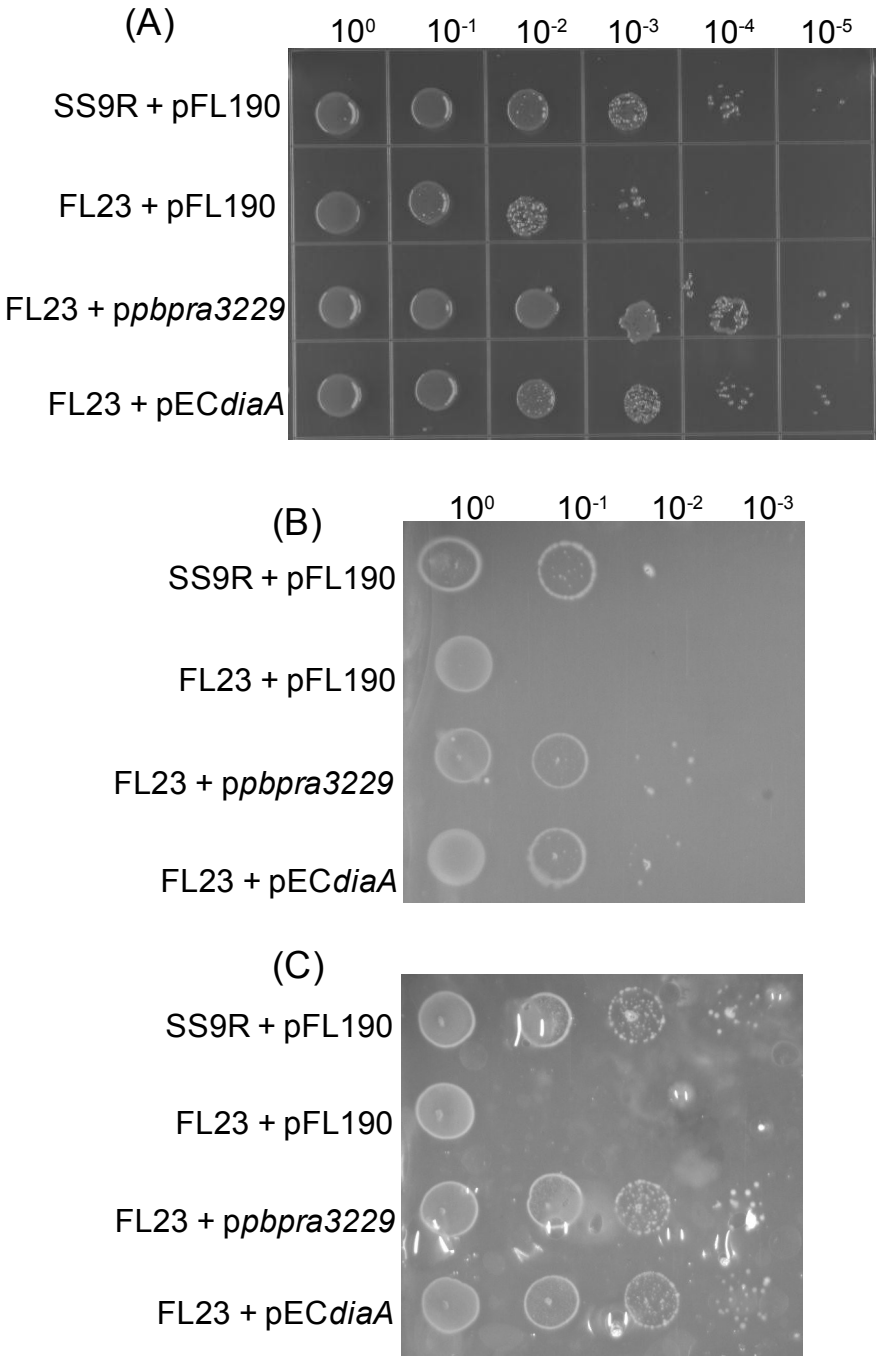


Figure 3-15. Complementation of the high pressure growth defect of *P. profundum* FL23 by EcDiaA. Strains were pre-grown in Marine broth, serially diluted and spotted on Marine agar (A) or LTG Marine agar (B & C) supplemented with arabinose (0.01% w/v). The plates were overlaid with LTG Marine agar (B & C only) and incubated at 15°C at 0.1MPa (A & B) or 28MPa (C) for 96 hours. Experiment was reproduced with similar results.

characteristic pressure defect that had been observed with the mutant and this was complemented by p*pbpra3229*, which restored growth to the same level as SS9R pFL190. However pEC*diaA* was also able to rescue the mutant to the same extent as p*pbpra3229*, suggesting that there was no difference between the functionality of PpDiaA and EcDiaA in *P. profundum* SS9 at 28MPa and 15°C. To confirm this the ability of PpDiaA and EcDiaA to rescue the abnormal cellular morphologies of FL23 was assessed by light microscopy at 15°C at 0.1, 28 and 45MPa. The introduction of pFL190 did not affect the morphology of SS9R, which formed mostly rods under all pressures (Figure 3-16 A-C). The morphology of FL23 was similarly unaffected, as the mutant formed a mixture of rods and filaments at 0.1 and 28MPa (Figure 3-16 D & E). The filamentation phenotype of FL23 was more dramatic at 45MPa (Figure 3-16 F), consistent with the high pressure sensitivity that initially characterised FL23 (Lauro *et al*, 2008). Introducing wild-type *P. profundum* SS9 *diaA* reverted the phenotype to wild-type under all pressures (Figure 3-16 G-I). As expected, introducing *E. coli diaA* into the mutant rescued the phenotype at 0.1MPa (Figure 3-16 J), since PpDiaA and EcDiaA have identical functions. The presence of *E. coli diaA* in the mutant also resulted in exclusively rod-shaped cells at 28MPa (Figure 3-16 K), confirming the phenotype complementation seen on agar (Figure 3-15 C). Even when the pressure was increased to 45MPa *E. coli diaA* was still able to fully complement the mutant phenotype in FL23 (Figure 3-16 L), providing further evidence that EcDiaA can function in *P. profundum* SS9 at high pressures. Taken together these data suggest that PpDiaA is not a pressure adapted protein, since EcDiaA can successfully complement the growth and morphology defects of FL23 at high pressure.

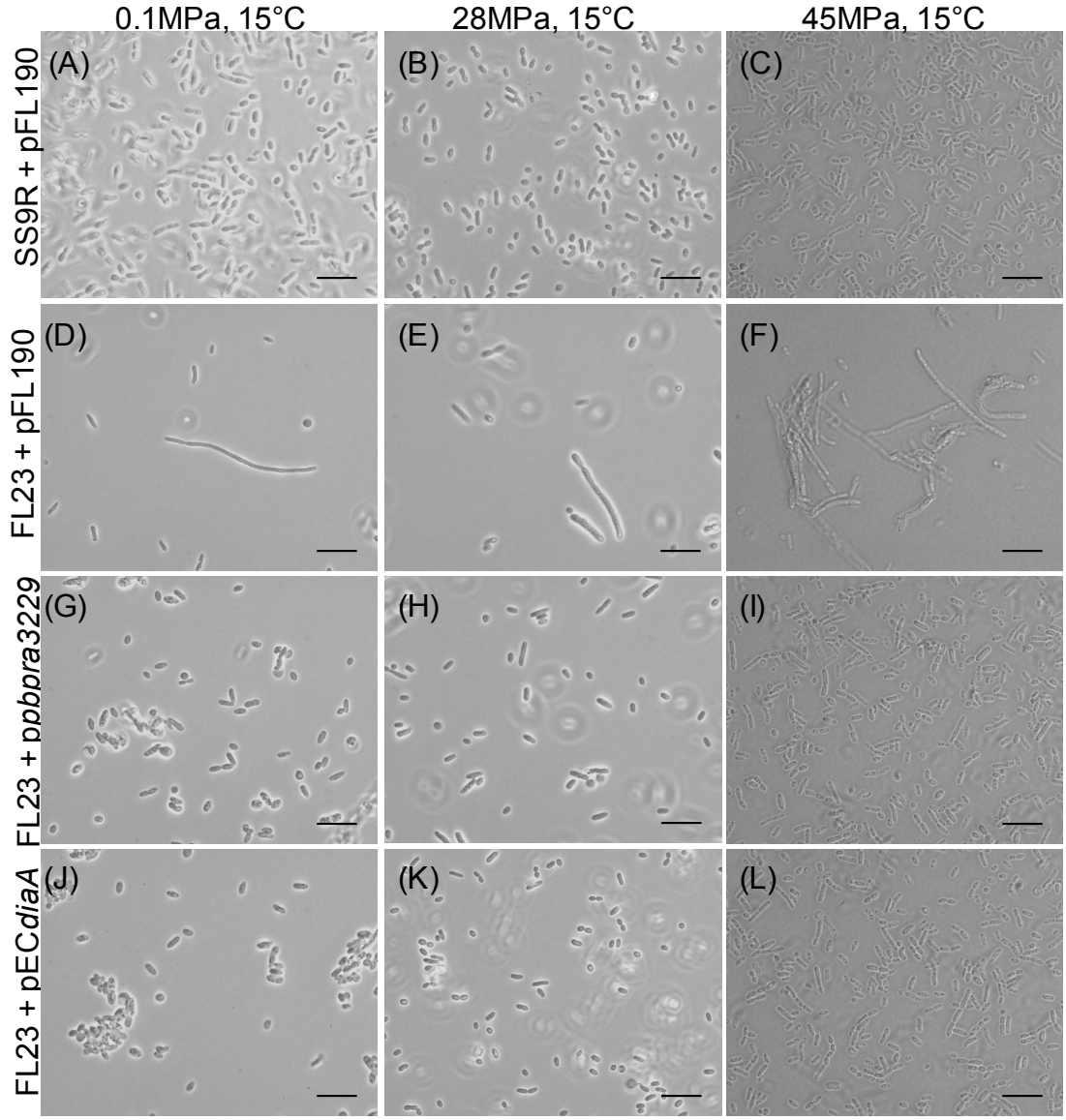


Figure 3-16. Complementation of the abnormal morphologies of *P. profundum* FL23 at high pressures by EcDiaA. Liquid cultures were grown to early stationary phase in Marine broth at the indicated conditions, and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. Scale bars represent 10µm. Experiment was reproduced with similar results.

3.4. A conditional pressure-sensitive *P. profundum* SS9 spherical mutant

During the mating of p*pbpra3229* into FL23 several exconjugants were isolated then screened for complementation of the FL23 mutant phenotype. The exconjugant described previously (Section 3.3.6) complemented the phenotype under all conditions examined and was considered to be a true complementing construct. One of the other exconjugants, termed ZE23 p*pbpra3229*, was complemented at atmospheric pressure but displayed a very unusual phenotype at high pressure.

3.4.1. Isolation and characterisation of the ZE23 spherical cell mutant

ZE23 p*pbpra3229* was initially thought identical to FL23 p*pbpra3229*. After isolation and purification of the exconjugant its growth on solid medium was assessed and compared to that of SS9R and FL23 carrying pFL190. ZE23 p*pbpra3229* had a colony forming ability that was comparable to that of SS9R pFL190 and was significantly improved over that of FL23 pFL190 at atmospheric pressure (Figure 3-17 A). It was therefore thought to be an identical clone of FL23 p*pbpra3229*, since it displayed the same phenotype under those conditions (Figure 3-15 A). However, when the phenotype of ZE23 p*pbpra3229* was assessed using the overlay method the colony forming ability of the construct was significantly impaired when the pressure was raised from 0.1 to 28MPa (Figure 3-17 B & C respectively). This was in marked contrast to the phenotype of the true FL23 p*pbpra3229* clone, where growth on solid medium is improved when the pressure is increased from 0.1 to 28MPa (Figure 3-15 B & C respectively).

The phenotype of ZE23 p*pbpra3229* was further characterised by growing

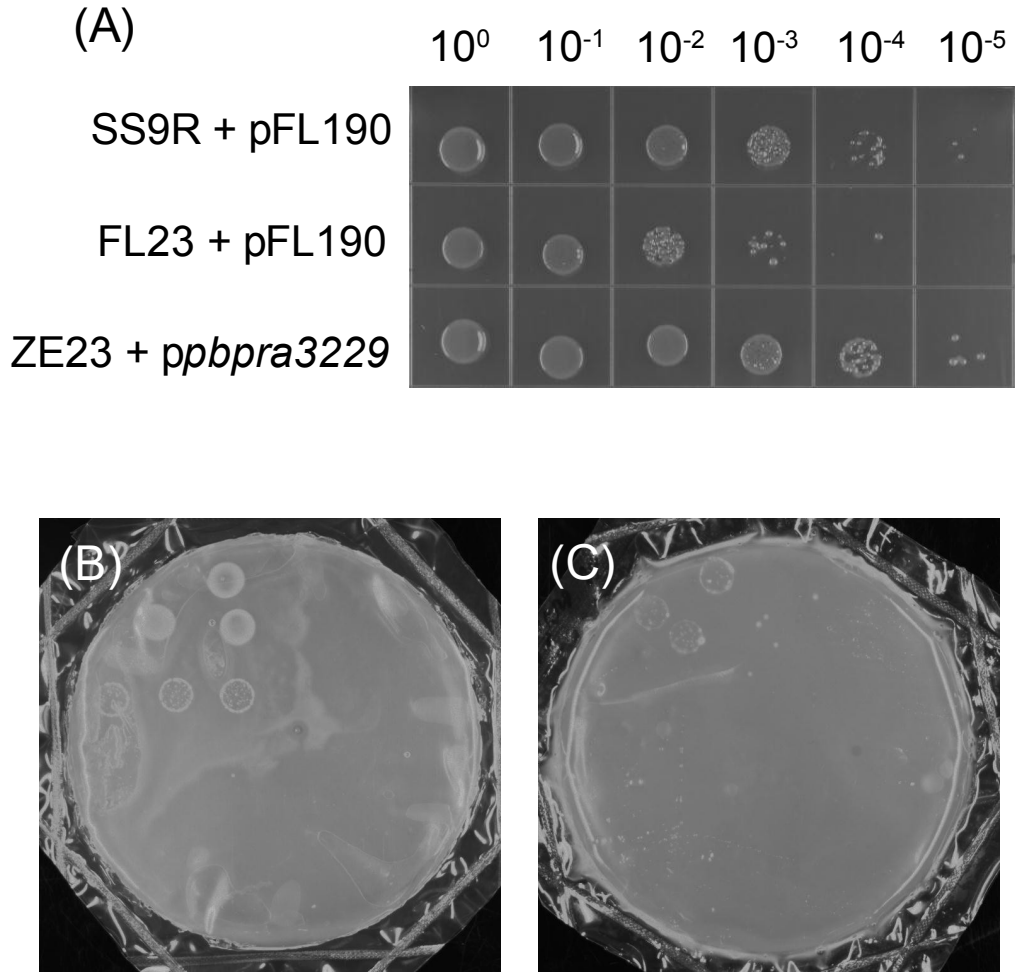


Figure 3-17. Isolation of the ZE23 spherical cell mutant. A: Strains were pre-grown in Marine broth, serially diluted and spotted on Marine agar supplemented with arabinose (0.01% w/v). The plate was incubated at 15°C and 0.1MPa for 96 hours. B & C: ZE23 + p*pbpra3229* was pre-grown in Marine broth, serially diluted and spotted on LGT Marine agar supplemented with arabinose (0.01% w/v).The plates were overlaid with LTG Marine agar and incubated at 15°C at 0.1MPa (B) or 28MPa (C) for 96 hours.

the strain in Marine broth at 0.1 and 28MPa at 15°C and comparing the cell morphologies to that of FL23 p*pbpra3229* under these conditions. At atmospheric pressure the morphologies of both strains were very similar and suggested that the cells were growing normally (Figure 3-18 A & B). As previously shown, FL23 p*pbpra3229* grew exclusively as short rods at 28MPa (Figure 3-18 C), indicating that the mutant phenotype of FL23 was fully complemented. However ZE23 p*pbpra3229* displayed a unique phenotype at 28MPa as no rods or filaments could be observed and the cells seemed to be spherical in shape (Figure 3-18 D). To confirm that these spheres were cells, the preparation was stained with the membrane dye FM4-64 and examined by fluorescence microscopy. The spheres that were observed with phase contrast were stained at their periphery by the membrane dye (Figure 3-18 E & F), providing evidence that the ZE23 p*pbpra3229* cells are spherical in shape.

3.4.2. ZE23 cells are spherical due to a chromosomal mutation

Comparing the phenotypes of FL23 p*pbpra3229* and ZE23 p*pbpra3229* it was hypothesised that a conditional mutation was present in the latter that was responsible for the formation of spherical cells at high pressure. The mutation had probably spontaneously arisen during triparental mating or the subsequent purification steps, therefore the mutation could be on either the chromosome or the plasmid. In order to check for its integrity, attempts were made to extract the plasmid from ZE23 p*pbpra3229* using a miniprep kit. However, these failed and subsequent attempts to extract pFL190 from SS9R were also unsuccessful. This suggested that *P. profundum* SS9 modifies plasmids in a way that makes them harder to recover using traditional plasmid extraction kits. Therefore, the plasmid was extracted by

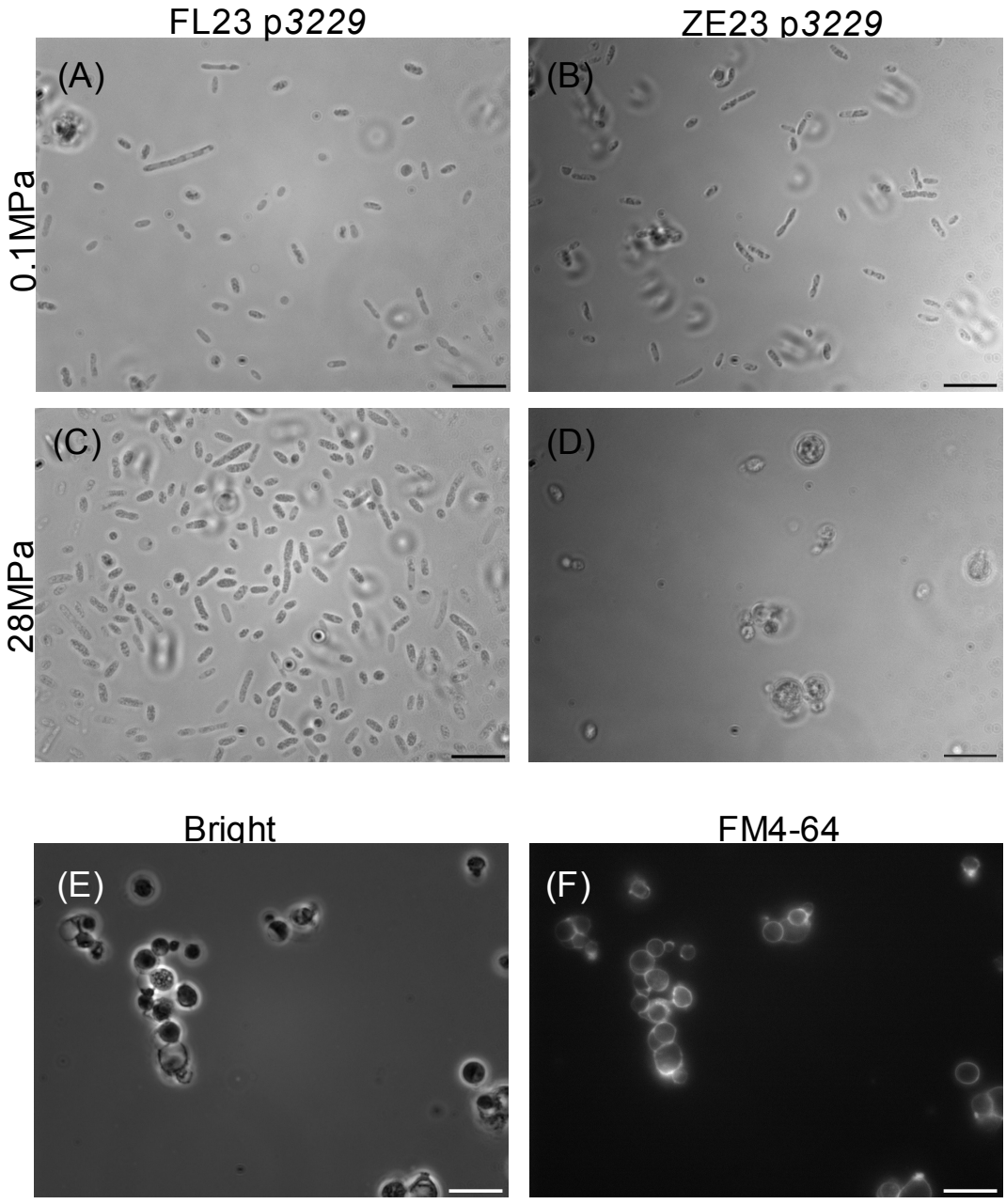


Figure 3-18. Cell morphology of the ZE23 conditional spherical mutant. A-D: Liquid cultures were grown to early stationary phase in Marine broth supplemented with streptomycin (100µg/ml) and arabinose (0.01% w/v) at the indicated pressure, and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. E & F: Cells were grow as above at 28MPa and stained with FM4-64 (2µM for 5 minutes) before visualisation with either phase contrast (E) or the TRITC filter (F). Scale bars represent 10µm.

using the alkaline lysis method (Section 2.3.3). The purified plasmid was then sequenced using the 190F primer (Table 2-5). The open reading frame was compared to the published sequence of the gene and no differences could be found. Additionally, the upstream sequence containing the promoter sequence was compared to the pFL190 sequence and was identical as well. This shows that the

To confirm that the spherical cell phenotype was independent of p*pbpra3229*, two separate experiments were designed. The first involved introducing the plasmid from ZE23 p*pbpra3229* into a fresh FL23 background and checking if the phenotype was transferred alongside the plasmid, while the second one required removing the plasmid and checking if cured ZE23 retained the mutant phenotype. To carry out the first experiment, the previously extracted plasmid was re-conjugated into regular FL23 by triparental mating. When the resulting construct was then grown in Marine broth at 28MPa and 15°C, it formed rod-shaped cells and no spheres were observed (Figure 3-19). This suggested that the spherical cell phenotype in ZE23 p*pbpra3229* is not due to the plasmid. The second experiment was carried out by inoculating ZE23 p*pbpra3229* in Marine broth without streptomycin at 0.1MPa and 15°C, growing until early stationary phase (40 hours) then diluting the culture 1:500. Under these conditions ZE23 grows similar to FL23 and does not form spherical cells (Figure 3-18 A & B) and the plasmid is not maintained due to the absence of streptomycin. The cycle was repeated a total of five times and the resulting culture was then plated on Marine agar with no antibiotic. One hundred colonies were picked and patched on Marine agar plates with or without streptomycin and incubated at 0.1MPa and 15°C for 96 hours (Figure 3-20 A & B). Five clones that did not grow on the streptomycin plate were picked from the non-streptomycin plate

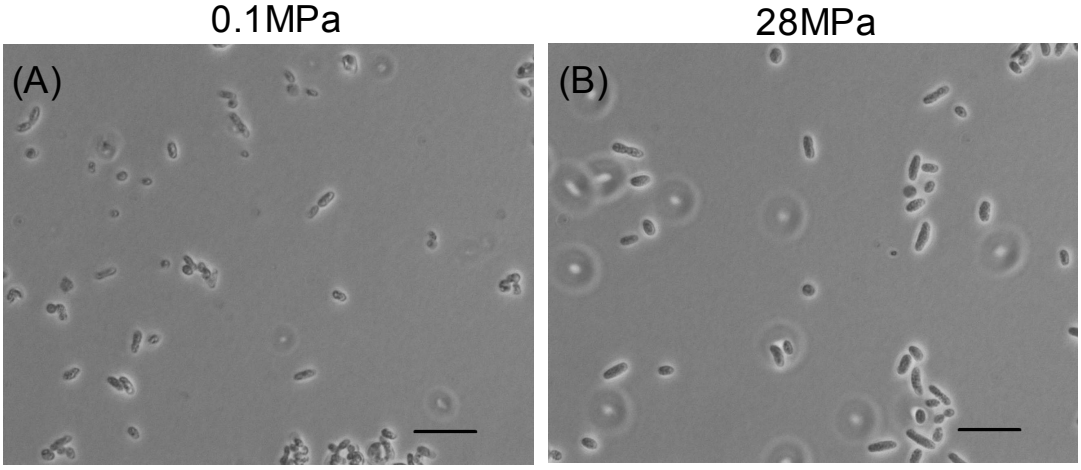


Figure 3-19. The spherical cell mutation is independent of the plasmid. The p*pbpra3229* plasmid was extracted from the ZE23 spherical mutant and conjugated into FL23. A liquid culture was then grown to early stationary phase in Marine broth supplemented with streptomycin (100µg/ml) and arabinose (0.01% w/v) at 0.1MPa (A) or 28MPa (B), and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. Scale bars represent 10µm.

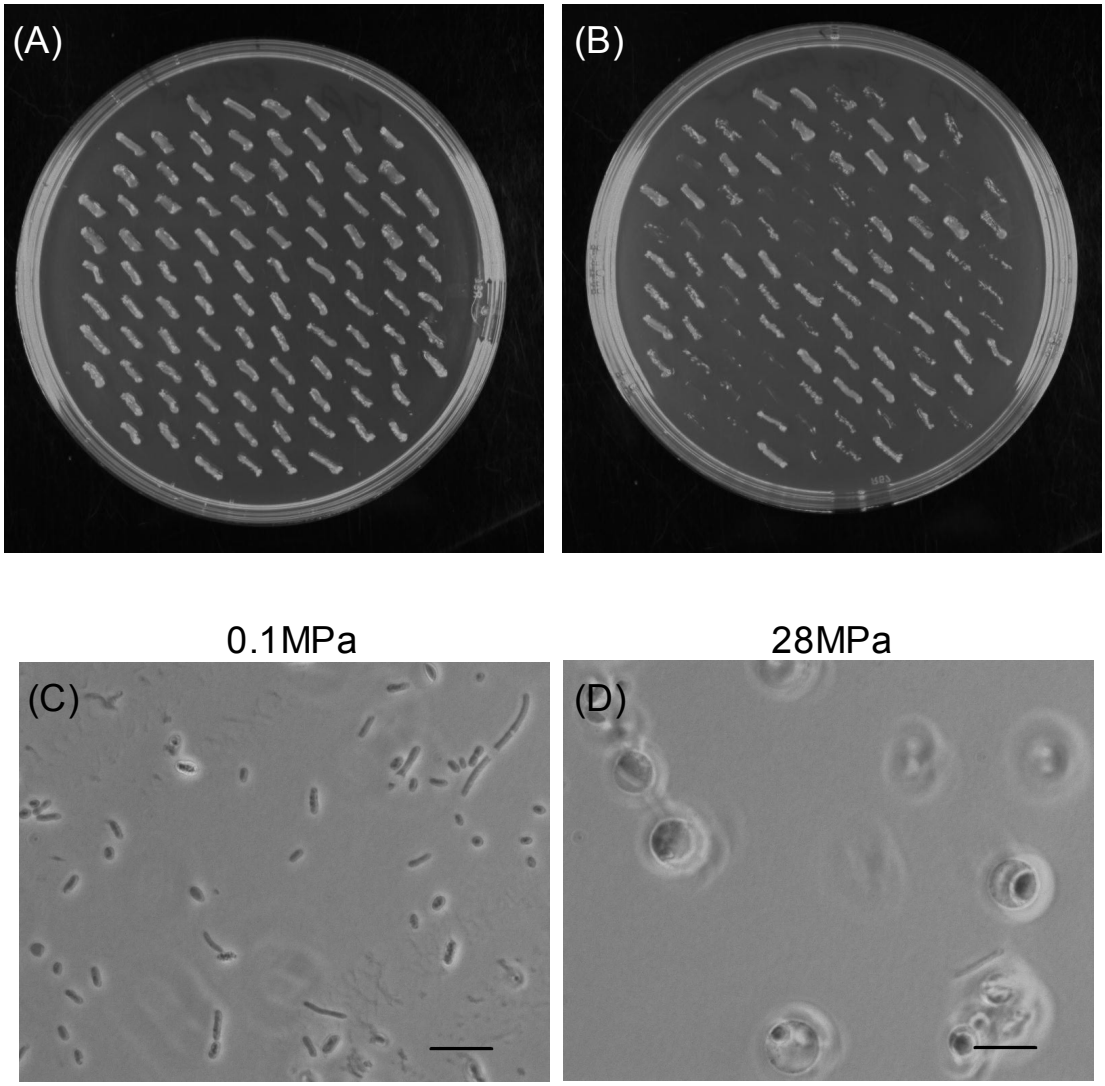


Figure 3-20. Curing ZE23 of the p*pbpra3229* plasmid. ZE23 p*pbpra3229* was grown in Marine broth with no streptomycin, diluted and regrown for 5 cycles. Cells were spread on Marine broth and 100 colonies were patched on Marine agar without antibiotic (A) or with 100µg/ml streptomycin (B). Five streptomycin-sensitive clones were picked from the first place and grown to early stationary phase in Marine broth at 0.1MPa (C) or 28MPa (D), and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. Scale bars represent 10µm.

and grown in Marine broth at 28MPa and 15°C. Microscopic examination of their cellular morphologies revealed that they still formed spherical cells (Figure 3-20 C & D), even though PCR with the 190F/190R primers showed they had been cured of the plasmid. To summarise, moving the plasmid does not carry over the spherical cell phenotype (Figure 3-21 A) and ZE23 forms spheres at 28MPa after being cured of the plasmid (Figure 3-21 B). This shows that the spherical cell phenotype of ZE23 is independent of the plasmid and most likely due to a chromosomal mutation.

3.5. The *diaA* operon

The *P. profundum* SS9 *diaA* gene is the third open reading frame in a cluster of four genes, the others being *pbpra3227*, *pbpra3228* and *pbpra3230. E. coli diaA* is likewise the third gene in a similar cluster with *yraM*, *yraN* and *yraP* (*E. coli diaA* was previously referred to as *yraO* before its involvement in DNA replication was known). The genomic organisation of the cluster suggested that the four genes formed an operon and therefore that all the genes could be involved in DNA replication.

3.5.1. The four genes form an operon in P. profundum SS9 and in E. coli

Table 3-1 describes the characteristics of the *diaA* gene cluster in *P. profundum* SS9 and *E. coli* K-12. Although both *diaA* genes have the same length none of the other pairs do, with extra base pairs present in the *E. coli* homologues. Additionally the amino acid identity of the other protein pairs is much lower than for PpDiaA and EcDiaA. This suggests that the major conservation in the cluster centers around *diaA*. In both organisms the 3' end of the first gene overlaps with the 5' end of

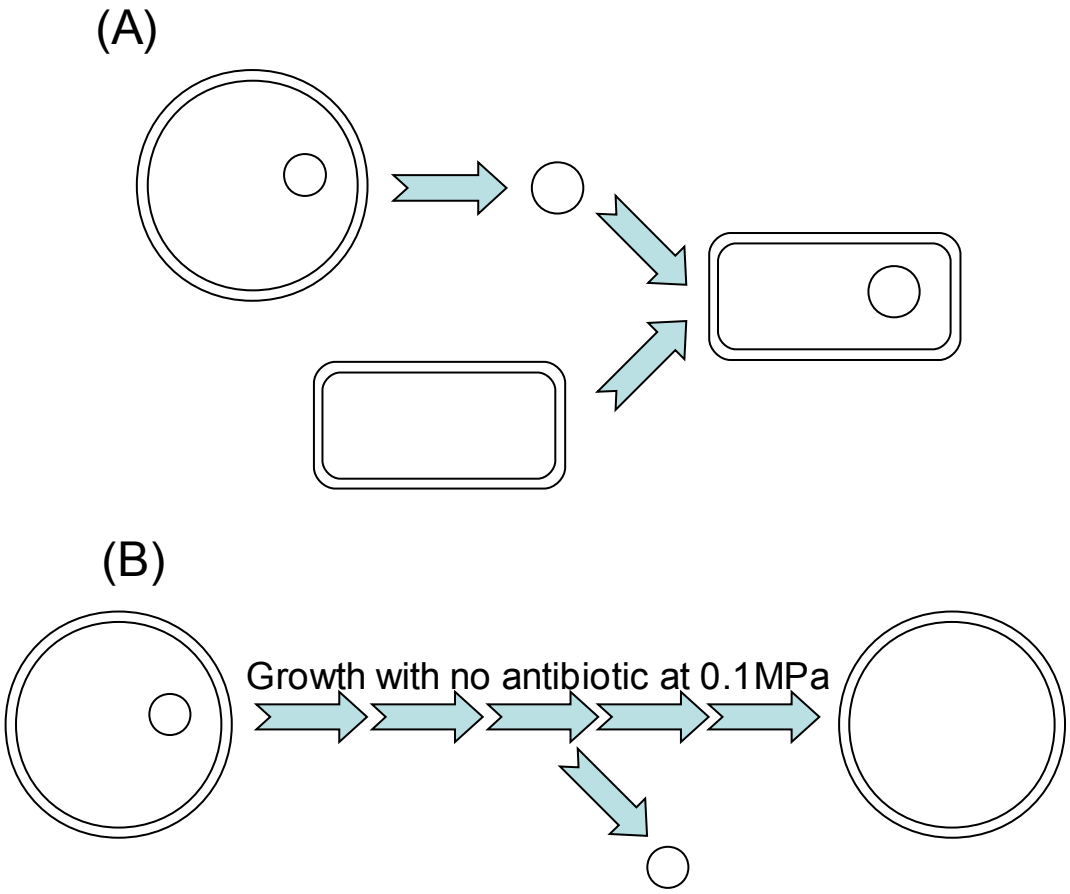


Figure 3-21. The mutation in the spherical mutant is chromosomal. A: The plasmid was extracted from ZE23 and conjugated into an original FL23 background, which maintained its rod shape at high pressure. B: ZE23 was grown for several cycles with no streptomycin until loss of the antibiotic resistance and therefore of the plasmid. The cells maintained their spherical shape at high pressure.

Table 3-1. The *P. profundum* SS9 and *E. coli diaA* operons

P. profundum operon		*E. coli* operon		Sequence identity
Gene	Length (bp)	Gene	Length (bp)	
pbpra3227	1842	*yraM*	2037	32%
pbpra3228	378	*yraN*	396	33%
pbpra3229	591	*diaA*	591	75%
pbpra3230	540	*yraP*	576	37%

the second one (Figure 3-22). All other gene ends are separated by a few base pairs, although both the overlap and the gaps are more extended in *E. coli* than in *P. profundum* SS9.

The overlap and small gaps suggested that the four genes in each organism form an operon. To test this hypothesis, total RNA was extracted and reverse transcribed using the pbpra3227+1506F/pbpra3230+290R primer pair for *P. profundum* SS9 and ECyraM+1674F/ECyraP+319R for *E. coli* (Table 2-5). The resulting cDNA was then amplified by PCR and analysed by agarose gel electrophoresis. The RT-PCR reactions from both *P. profundum* SS9 and *E. coli* RNA produced an amplicon of around 1.6kb (Figure 3-23 A & B respectively), the expected size for the primer pairs that were used, which shows that the four genes can be amplified together from the RNA samples. The control reaction lacking reverse transcriptase did not produce an amplicon, thereby confirming that the RNA sample is not contaminated with DNA and that the gene cluster in both *P. profundum* SS9 and *E. coli* is transcribed as a single polycistronic mRNA.

3.5.2. YraM and YraP are involved in DNA replication through DnaA

Since *diaA* was shown to be in an operon with three other genes in both *P. profundum* SS9 and in *E. coli*, it was likely that these other genes coded for proteins that interacted with DiaA or were also involved in DNA replication. However since the amino acid identities between the *P. profundum* SS9 and the *E. coli* proteins were not as high as PpDiaA and EcDiaA it was necessary first to test if a protein from one organism could substitute for its respective homologue in the other organism. Since no published data is available on *yraM*, *yraN* or *yraP* it was decided to test if any of

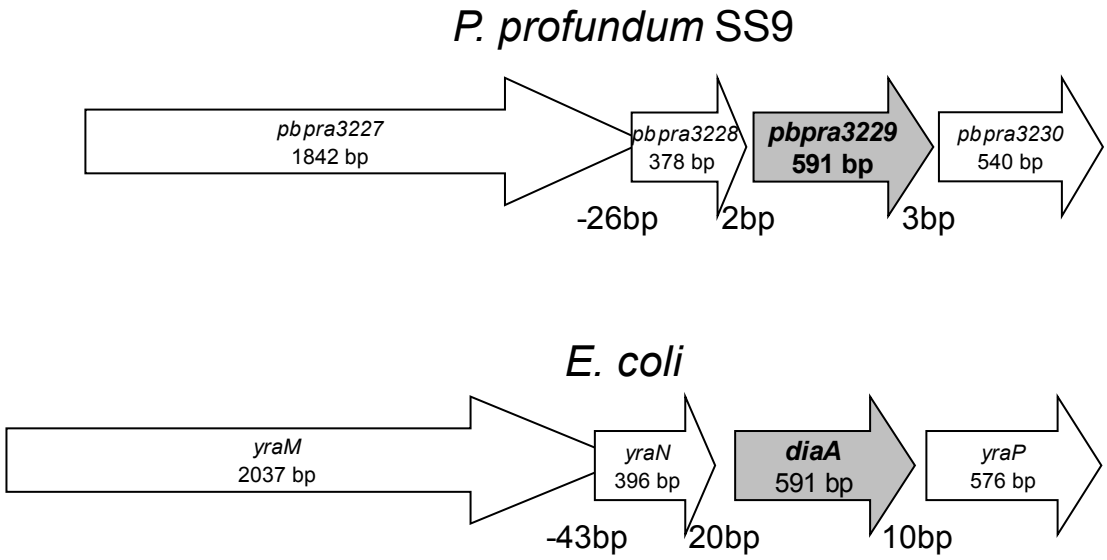


Figure 3-22. The *pbpra3229* and *diaA* gene clusters. Schematic representation of the surrounding genomic regions of *pbpra3229* (A) and *diaA* (B) showing overlaps and gaps between the genes.

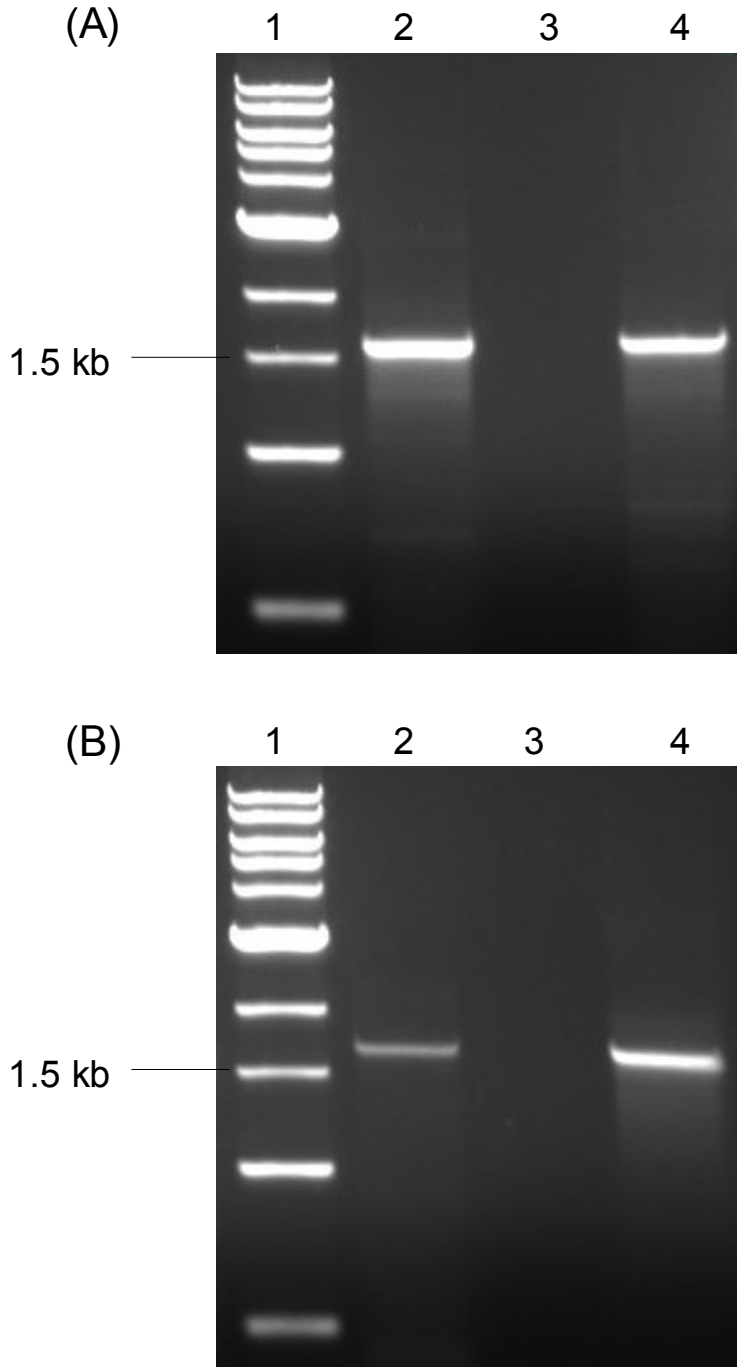


Figure 3-23. RT-PCR analysis of the *P. profundum* and *E. coli diaA* operons. Total RNA from *P. profundum* (A) and *E. coli* (B) was extracted and reverse transcribed, the cDNA was amplified then analysed by agarose gel electrophoresis. The lanes have the 1kb DNA ladder (1), the RT-PCR reaction (2), a negative control with no reverse transcriptase (3) and a positive control with DNA instead of RNA (4).

the mutants had a growth phenotype that could be complemented by the introduction of its *P. profundum* SS9 homologue. Deletion mutants of *yraM* and *yraP*, JW3116 and JW3119 respectively, were available as part of the Keio collection (Baba *et al*, 2006). In these mutants the targeted genes are replaced by a kanamycin cassette. Attempts at creating a *yraN* deletion mutant had proved unsuccessful and it was thought to be an essential gene (Baba *et al*, 2006). The Δ*yraM*::kan and Δ*yraP*::kan mutations were moved into the KH5402-1 background using P1 transduction to generate strains ZE3116 and ZE3119. The mutants and the parent were then inoculated and their growth on agar was assessed alongside NA141, the *diaA* mutant in the same background, whose colony forming ability has been shown to be indistinguishable from the parent (Figure 3-11). Both ZE3116 and ZE3119 grew to the same dilution as the parent and the *diaA* mutant (Figure 3-24), suggesting that loss of YraM or YraP does not affect growth and viability in *E. coli*.

Since the single mutants did not display a growth phenotype another method was needed to test for the ability of the *P. profundum* SS9 proteins to complement their *E. coli* homologues. It was hypothesised that if either *yraM* or *yraP* coded for a protein involved in promoting DNA replication, they could act as intergenic suppressors of the *dnaAcos* mutation in a manner similar to *diaA*. To test this, the P1 lysates from JW3116 and JW3119 were transduced into the *dnaAcos* strain NA001 to generate strains ZE3116cos (*dnaAcos* Δ*yraM*::kan) and ZE3119cos (*dnaAcos* Δ*yraP*::kan) respectively. The growth of these mutants was then compared to that of NA001 to assess the ability of the *yraM* and *yraP* deletions to suppress the cold-sensitivity of *dnaAcos*. The introduction of either mutation into the *dnaAcos* background restored growth at 30°C in a similar manner to the *diaA* mutation and up

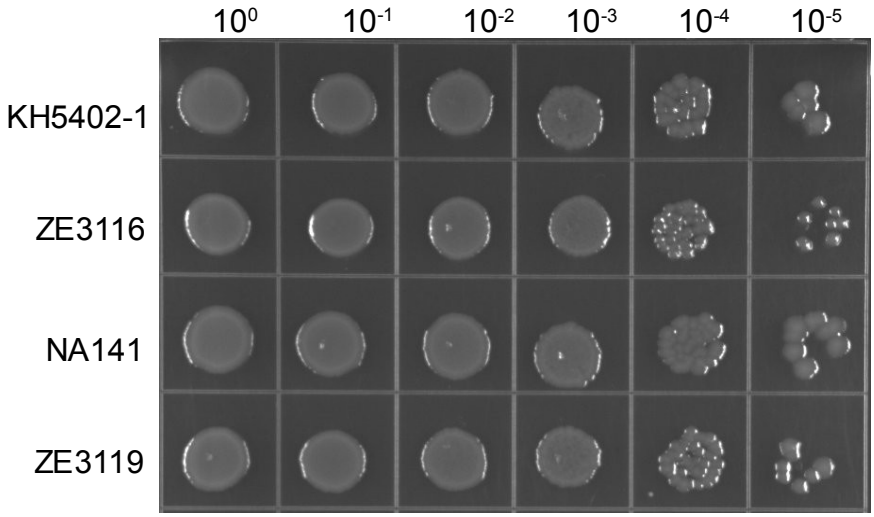


Figure 3-24. Growth of the *E. coli yraM* and *yraP* mutants on LB agar. The *E. coli* parent (KH5402-1) and the *YraM* (ZE3116), *diaA* (NA141) and *yraP* (ZE3119) mutants were grown overnight in LB broth supplemented with thymine, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted on LB agar supplemented with thymine. Plates were incubated at 37°C overnight.

to the same level as the parent strain (Figure 3-25 A). As with *diaA*, introducing either mutation did not affect growth at 42°C (Figure 3-25 B). This indicates that deleting *yraM* or *yraP* suppresses the *dnaAcos* phenotype at 30°C and suggests that YraM and YraP are involved in initiation of DNA replication through direct or indirect interaction with DnaA. Complementation with wild-type *yraM* is necessary to confirm that the suppression phenotype observed ZE3116cos is not due to a polar effect on *diaA*.

3.5.3. Loss of YraM or YraP does not directly affect timely initiation of DNA replication

The ability of *yraM* and *yraP* to suppress the *dnaAcos* cold-sensitive phenotype suggested a role for their gene products in DNA replication. Since flow cytometry has proven to be a reliable method to observe the effect of mutations of initiation of replication, exponential cultures ZE3116 (Δ*yraM*::kan) and ZE3119 (Δ*yraP*::kan) were treated, stained, analysed by flow cytometry and compared to the parent strain KH5402-1. Surprisingly both mutants showed a profile similar to that of the parent strain with most cells having 2 or 4 chromosomes, as seen by the presence of two sharp peaks at a fluorescence intensity of 200 and 400 respectively (Figure 3-26). This shows that the loss of YraM or YraP does not directly affect DNA replication and suggests that their function is different from that of DiaA and is dispensable under the conditions observed.

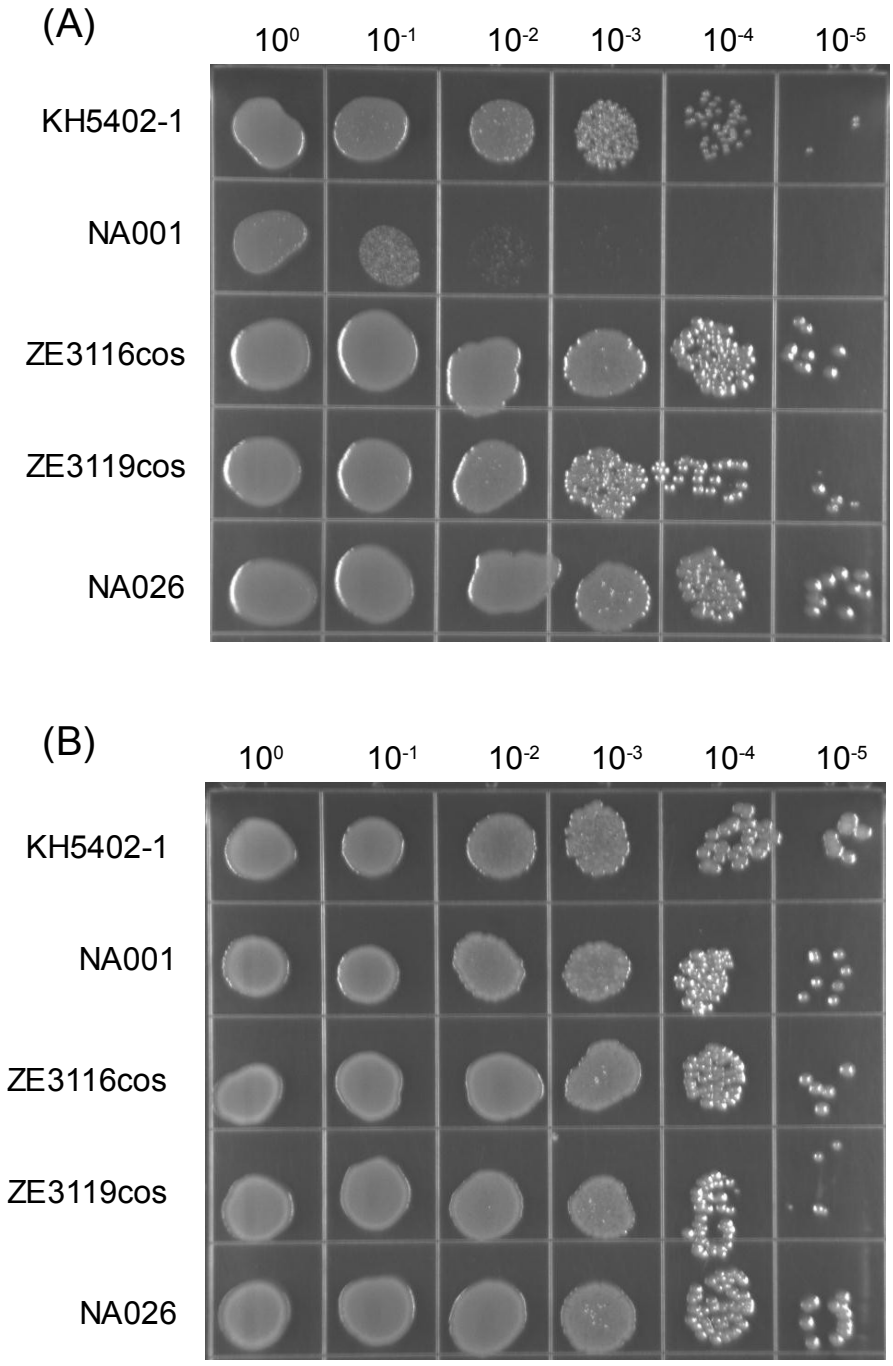


Figure 3-25. Suppression of the cold sensitivity of *E. coli dnaAcos* by deletion of *yraM* or *yraP*.
The *E. coli* parent (KH5402-1), *dnaAcos* mutant (NA001) and *dnaAcos yraM* (ZE3116cos), *yraP* (ZE3119cos) and *diaA* (NA026) double mutants were grown overnight in LB broth supplemented with thymine, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted on LB agar supplemented with thymine. Plates were incubated at 30°C (A) or 42°C (B) overnight.

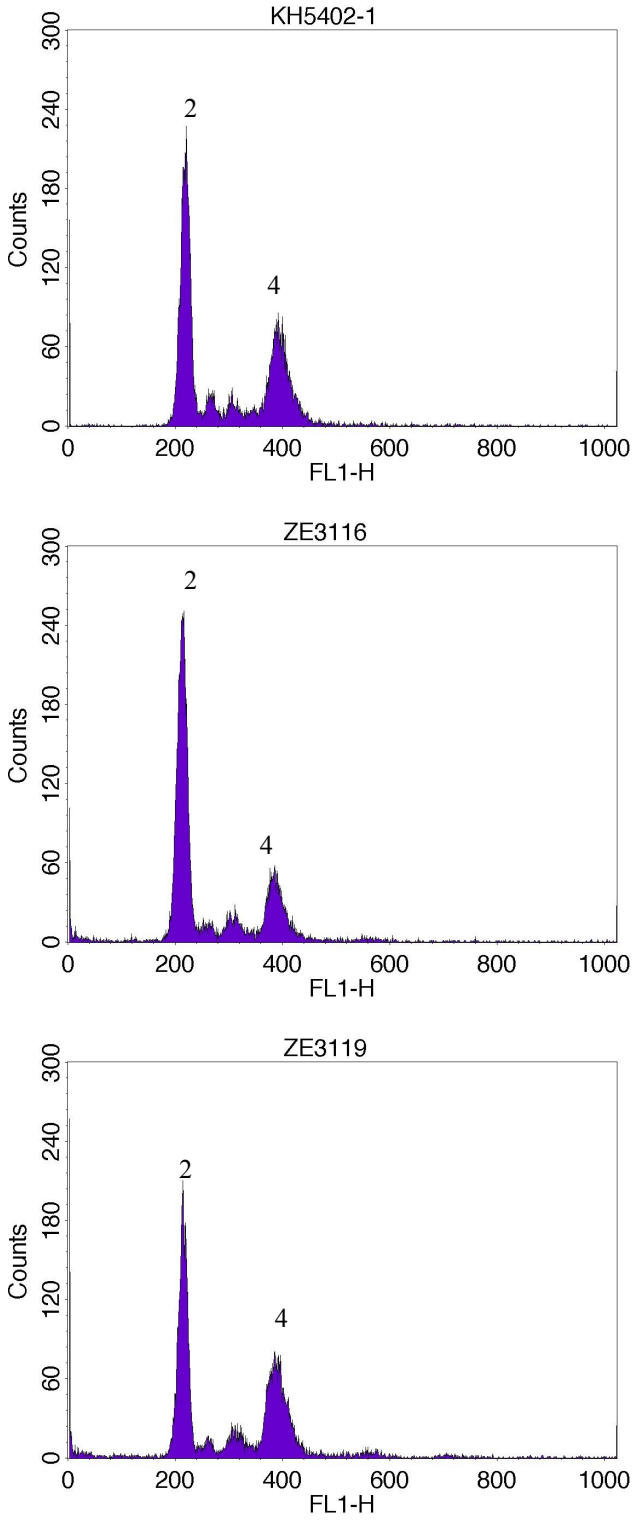


Figure 3-26. Initiation of DNA replication in *yraM* and *yraP* mutants. The *E. coli* parent (KH5402-1), *yraM* (ZE3116) and *yraP* (ZE3119) mutants were grown at 30°C in LB until mid-exponential phase, treated with rifampicin and cephalexin, fixed in 70% ethanol then the DNA was stained with Sytox Green and analysed by flow cytometry. Chromosome number is indicated above the peaks.

3.5.4. Generating pbpra3227, pbpra3228 and *pbpra3230* mutants in *P. profundum SS9*

The involvement of *pbpra3229* in both high pressure adaptation and DNA replication in *P. profundum* SS9 as well as the confirmation that it is part of an operon suggested that the other genes in that operon could be involved in both of these processes as well. Suppression of *E. coli dnaAcos* by the *yraM* and *yraP* mutants provided further evidence of this involvement. Since the main focus of this study is on the link between DNA replication and deep-sea adaptation, it was decided to focus on mutants in the operon genes in *P. profundum* SS9 rather than *E. coli*.

To this end attempts to create mini-Tn*5* insertion mutants in *pbpra3227*, *pbpra3228* and *pbpra3230* were made. The pEE3 suicide vector was chosen because it was designed specifically for use in *P. profundum* SS9 and includes a TA cloning site within *lacZ* for simple cloning and blue/white screening (Lauro *et al*, 2005). Internal fragments from the *pbpra3227*, *pbpra3228* and *pbpra3230* genes were amplified by PCR and cloned into pEE3 to generate plasmids pEE3227, pEE3228 and pEE3230 respectively. Several attempts were then made to conjugate the vectors into SS9R and generate insertional mutants but no exconjugants could be isolated for any of the plasmids. Due to time constraints further attempts at refining the conjugation efficiency could not be made. However the constructs are available and further attempts at optimising the conjugation can be made.

3.6. Intact RctB is required for high pressure growth of *P. profundum* SS9

Like all *Vibrionaceae*, *P. profundum* SS9 has two chromosomes with distinct

replication requirements. In *V. cholerae* the two initiation mechanisms are independent of each other (Duigou *et al*, 2006). As mentioned previously (Section 1.4.3), DnaA is the initiator protein for chrI and the equivalent protein for chrII is RctB. Although *rctB* is an essential gene, the *P. profundum* FL31 mutant carries a mini-Tn*5* insertion in *pbprb0001*, the *P. profundum* SS9 homologue of *rctB*, that only disrupts the C-terminus of the protein and probably encodes a partially functional protein (Lauro *et al*, 2008). Like FL23, FL31 was isolated as pressure-sensitive and it is hypothesised that the disruption of initiation of chrII replication is responsible for the mutant's pressure sensitivity, in contrast to FL23 where disruption of initiation of chrI replication is thought to be the culprit.

In order to determine if FL31 formed abnormal morphologies similar to FL23, SS9R and FL31 cells were cultured at 0.1, 28 and 45MPa at 15°C and examined by phase contrast microscopy. Both strains were mostly rod-shaped at 0.1MPa and 15°C with the presence of occasional short filaments (Figure 3-27 A & B). As expected SS9R was rod-shaped at 28MPa and 15°C, although surprisingly FL31 was similarly rod-shaped under these conditions (Figure 3-27 C & D respectively). At 45MPa and 15°C SS9R was still rod-shaped, but FL31 formed a mixture of rod, filaments and what appeared to be small spheres or minicells (Figure 3-27 C & D respectively), consistent with the high pressure sensitivity seen at 45MPa (Lauro *et al*, 2008). This shows that FL31 grows as normal rod-shaped cells under the optimal growth conditions for *P. profundum* SS9 but forms aberrant morphologies when the pressure is increased above the optimum, in contrast to FL23 which forms abnormal morphologies even at the optimal pressure (Figure 3-6).

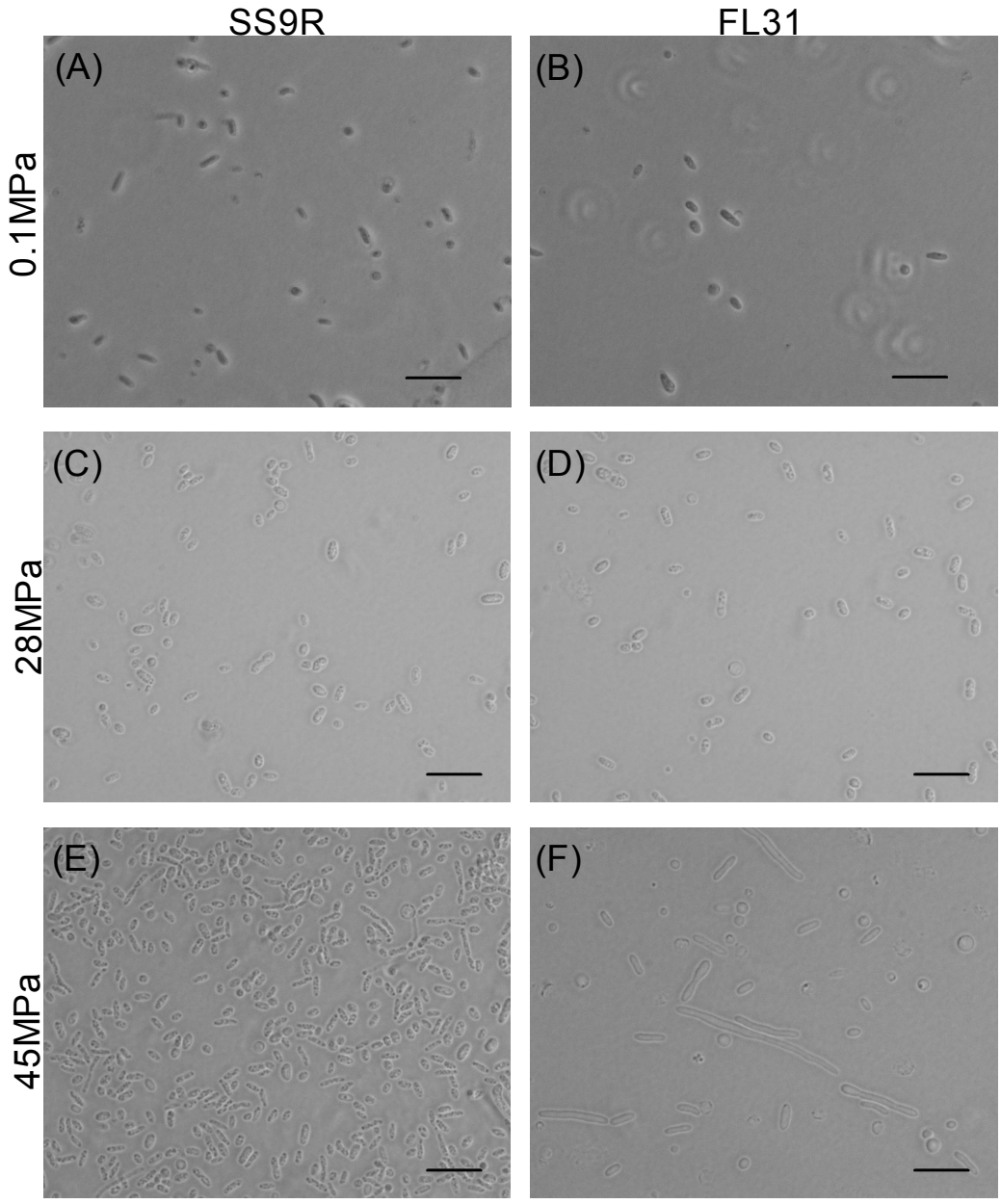


Figure 3-27. Cell morphology of FL31 by phase contrast microscopy. The *P. profundum* parent (SS9R) and *rctB* mutant (FL31) were grown to early stationary phase in Marine broth at the indicated pressure, and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. Scale bars represent 10µm. Experiment was reproduced with similar results.

3.7. Discussion

FL23 is a mutant of *P. profundum* SS9R with a mini-Tn*10* insertion in the *pbpra3229* gene, whose product was annotated as a GmhA. FL23 was initially isolated as pressure-sensitive in liquid medium at 45MPa and 17°C. This study determined that Pbpra3229 is not a GmhA homologue but a *P. profundum* SS9 DiaA.

3.7.1. Growth phenotype of FL23 in liquid versus solid medium

FL23 is pressure sensitive at 45MPa, but this sensitivity is less pronounced at 28MPa, the optimal pressure for growth of *P. profundum* SS9. FL23 has a growth defect on solid medium under a range of temperatures at atmospheric pressure but shows a radical loss of pressure adaptation using the low gelling temperature agar overlay method. This mutants also forms a mixture of rods and cells with aberrant cellular morphologies when observed using both phase contrast and electron microscopy.

Although SS9R is rod-shaped at the optimal temperature and pressure (28MPa and 15°C), FL23 filaments and forms abnormal morphologies under these conditions. This morphological defect can account for the growth phenotypes observed in liquid and solid media. It should be noted however that the severity of the phenotype varies widely between these two media. In liquid medium the mutant exhibits a growth defect compared to the parent strain under most conditions tested, which is exacerbated at 0.1MPa and 4°C, but the defect is much more drastic on solid medium, as growth of the mutant is reduced by two orders of magnitude at 0.1MPa and 15°C and up to five orders of magnitude at 28MPa and 4°C.

One possible explanation is that mutant cells are capable of growth and

elongation but cannot divide, which would lead to an increase in optical density but not in number of cells and of colony forming units. However this hypothesis cannot account for the massive reduction in colony forming units at 28MPa and 4°C, especially since the optical density of a liquid culture still increases exponentially under these conditions at a point where it would be expected that the existing cells would have already lysed if they were filamentous. An alternative hypothesis is that the *P. profundum* SS9 employs distinct mechanisms when in planktonic culture as opposed to attached on a surface such as agar and that, while disruption of *pbpra3229* affects both mechanisms, its effect on surface-attached growth is much more drastic and is exacerbated by environmental stresses such as high pressure and low temperature. This hypothesis could be tested by assessing the growth or the ability of *P. profundum* SS9 to form biofilms on a number of different surfaces.

In experiments where pFL190 and derivatives were used in *P. profundum* SS9, the presence of glucose in Marine agar did not seem to affect induction of the pFL190 P_{BAD} promoter by arabinose. Since *P. profundum* SS9 had already been shown to be unable to metabolise arabinose (Nogi *et al*, 1998) this suggested that catabolite repression of the P_{BAD} promoter (Newman and Fuqua, 1999) is absent in *P. profundum* SS9.

3.7.2. Pbpra3229 is a DiaA homologue and not a GmhA homologue

Pbpra3229 had been annotated as a putative phosphoheptose isomerase due to its homology to *E. coli* GmhA and the presence of region homologous to the SiS domain within its sequence. However, SDS-PAGE analysis of the LPS from FL23 does not show a truncated LPS, a phenotype that typically characterises the *E. coli*

gmhA mutant and the subsequent loss of the inner core of the LPS. Additionally, Pbpra3229 has higher identity to EcDiaA, a DnaA-binding protein involved in the timely initiation of DNA replication. DiaA also contains a region homologous to the SiS domain, although the significance is unclear as EcDiaA has been shown to be unable to bind phosphoheptose and cannot bind DNA directly (Katayama *et al*, 2007) Although the 3D structures of EcDiaA and GmhA are highly similar and the homology model of PpDiaA is comparable to both, sequence alignment shows that PpDiaA and EcDiaA share much higher homology (75%) with each other than either does with GmhA (45% and 40% respectively).

The LPS analysis as well as the protein alignment suggested that Pbpra3229 is a homologue of DiaA rather than GmhA. This was evidenced by the ability of wild-type *E. coli diaA* to complement the morphological defects of FL23 cells as well as the colony forming defect exhibited on solid agar, at both 0.1 and 28MPa. Conversely, wild-type *pbpra3229* complemented the growth phenotype of an *E. coli dnaAcos diaA*::Tn*5* double mutant by restoring the cold sensitivity that is suppressed in the *dnaAcos* background by disruption of *diaA*. Conclusive evidence that Pbpra3229 is a *P. profundum* SS9 homologue of DiaA was provided by flow cytometry analysis, which clearly showed the ability of Pbpra3229 to restore timely initiation of DNA replication in an *E. coli diaA* mutant to the same extent as the wild-type *E. coli* protein.

Attempts at quantifying the DNA and chromosomal content of *P. profundum* SS9R by flow cytometry were unsuccessful. Although this could be due to the presence of two chromosomes in SS9R, the same methodology used in *E. coli* has been successfully applied to *V. cholerae*, another two-chromosome organism

(Duigou *et al*, 2006). Instead the problem could be due to the resistance of SS9R to rifampicin, one of the antibiotics required to treat the cells prior to analysing them by flow cytometry. It should also be noted that in the experiments carried out with both *E. coli* and *V. cholerae* the cells were grown and treated at 30℃ or above. It is not known if the modes of action of rifampicin and cephalexin are affected at lower temperature, which is required for growth of *P. profundum* SS9. Another problem is that FL23 filaments and forms abnormal morphologies under all conditions observed and those would interfere with the flow cytometry. Rifampicin resistance can be avoided by using the wild-type *P. profundum* SS9 strain. At present there is no simple method to transfer mutations between *P. profundum* SS9 strains but if one is developed this would allow the *pbpra3229*:Tn*10* mutation to be moved into SS9 and would make further experimentation with flow cytometry in *P. profundum* SS9 more worthwhile. Another method that has been successfully used in *V. cholerae* involves using *tet* operator sequence arrays inserted near the origin of replication, which allows counting of the number of origins per cell and therefore assessing the state of DNA replication in a particular strain (Duigou *et al*, 2006). One advantage of operator sequence arrays is that they do not require cells in a culture to be of uniform shape and could therefore be used with mutants that form aberrant morphologies such as FL23.

3.7.3. The diaA operon in P. profundum SS9 and E. coli

The *pbpra3229* gene is in a cluster with three other genes. In order to confirm that the phenotype observed in FL23 is due to the disruption of *pbpra3229* and not to downstream polar effects of the mini-Tn*10*, the mutation was successfully

complemented with wild-type *pbpra3229*. None of the genes were annotated and nothing was known about their potential gene products. Their homologues in *E. coli* are in a similar cluster with *diaA* and there is likewise nothing known about their function. The four genes are clustered together very tightly and there is some overlap in both organisms, which suggested that the genes form an operon and are also involved in DNA replication.

This study has shown that *diaA* is transcribed as part of a four-gene operon in both *P. profundum* SS9 and *E. coli*. The ability of *yraM* and *yraP* deletions to suppress the cold sensitivity of *E. coli dnaAcos* suggests that the two genes are involved in DNA replication through interaction with DnaA. However neither deletion resulted in disruption of initiation of replication as seen by flow cytometry. This suggests that YraM and YraP are indirectly involved in the process. Alternatively the proteins are not required for initiation but may promote replication only under specific circumstances such as compensating for the loss of function in other replication proteins. This may explain why DiaA, which is required for timely initiation of replication, is much more highly conserved than the other three genes.

As with PpDiaA and EcDiaA, mutants in the other operon genes in *P. profundum* SS9 may have more severe phenotypes than their *E. coli* counterparts due to the presence of the second chromosome. Attempts at creating the *P. profundum* SS9 mutants have so far been unsuccessful but the constructs are available and tweaking the conjugation conditions may increase the efficiency and lead to successful mutagenesis. By adapting flow cytometry or operator sequence arrays to work in *P. profundum* SS9, the effect of the mutations on DNA replication can be directly observed and if these are different from their *E. coli* counterparts this will

yield great insight into the difference in initiation of replication between organisms with one or two chromosomes.

3.7.4. The P. profundum SS9 spherical cell mutant

During the conjugation experiments that preceded complementation of FL23, a remarkable mutant was isolated. ZE23 was the product of an attempt at conjugating p*pbpra3229* into FL23. ZE23 p*pbpra3229* seemed to grow normally at atmospheric pressure but formed lethal spherical cells at 28MPa, a phenotype that had never been observed with *P. profundum* SS9. Initial suspicions that the plasmid had undergone a mutation were quickly dispelled when sequencing of the plasmid insert as well as its upstream region revealed no mutations. Extracting p*pbpra3229* and conjugating the plasmid into a fresh FL23 background does not reintroduce the spherical cell phenotype. Furthermore p*pbpra3229* can be cured from ZE23 and this does not affect the phenotype either. ZE23 is therefore thought to carry a chromosomal mutation that is responsible for the unusual phenotype at high pressure.

Spherical cell mutants have been well-studied in *E. coli*. The normally rod-shaped *E. coli* cells can become spherical either by treatment with the β-lactam antibiotic mecillinam (Long *et al*, 1978) or by inactivation of genes whose products are involved in maintenance of the rod shape such as *rodA*, *pbpA* or a member of the *mre* gene cluster (Ogura *et al*, 1989; Wachi *et al*, 1987). One hypothesis is that ZE23 acquired a mutation in a homologue of one of these genes or in another gene involved in cell shape maintenance in *P. profundum* SS9. The mutation does not affect the function of the protein at atmospheric pressure but the mutant protein may be much more sensitive to the stress of high pressure, resulting in a conditional

mutant with normal morphology at 0.1MPa but lethal spherical cells at 28MPa.

None of the genetic tools currently available with *P. profundum* SS9 allow easy identification of unmarked chromosomal mutations. As more tools are developed it could become possible to pinpoint the location and exact nature of this mutation. Doing so may yield valuable insights into the relation between cell shape and pressure adaptation.

3.7.5. RctB and initiation of replication with multiple chromosomes

Unlike FL23, the *E. coli diaA* mutant does not exhibit a growth defect or abnormal cellular morphologies (Ishida *et al*, 2004). This suggests that *E. coli* can cope with the loss of DiaA much more readily than *P. profundum* SS9 with that of Pbpra3229. *E. coli* has only one chromosome whereas *P. profundum* SS9, like all *Vibrionaceae*, has two (Vezzi *et al*, 2005). In *V. cholerae*, which is considered a model for all other *Vibrionaceae*, the mechanisms involved in initiating replication at each chromosome are independent of each other even though initiation of replication of the two chromosomes is synchronous (Duigou *et al*, 2006). Therefore the loss of Pbpra3229 would disrupt timely initiation of replication in Chromosome I but would not affect Chromosome II. With loss of synchrony between the two chromosomes some of the daughter cells may end up with only one chromosome, potentially leading to the abnormal morphologies that were observed. This may be exacerbated by high pressure, an environmental stress that *P. profundum* SS9 has to deal with in its native niche in the deep-sea, and may be the reason behind the loss of pressure adaptation seen in FL23.

One of the components involved in replication of chrII in *V. cholerae* is RctB,

a multifunctional protein and the initiator of chrII replication (Egan and Waldor, 2003). Although *rctB* is an essential gene, *P. profundum* FL31 carries a mini-transposon insertion in *pbprb0001*, the *rctB* homologue, that is thought to encode a partially functional protein (Lauro *et al*, 2008). Although FL31 did not form abnormal morphologies at 28MPa it heavily filamented at 45MPa. DiaA and RctB are involved in promoting initiation of DNA replication in chrI and chrII respectively and loss of either protein in *P. profundum* SS9 results in high pressure sensitivity and formation of abnormal morphologies at high pressure. This points to synchronous initiation of replication of the two chromosomes being vital under deep-sea conditions in *P. profundum* SS9.

3.7.6. The SeqA homologue in P. profundum SS9

FL28 is a *P. profundum* SS9 mutant with a mini-Tn*10* insertion in *pbpra1039*, which encodes a putative homologue of *E. coli* SeqA (55% protein identity). In *E. coli*, SeqA negatively regulates DNA replication while DiaA enhances replication, but both are required for timely initiation of replication (Messer, 2002; Ishida *et al*, 2004). FL28 was described as a "pressure-enhanced" mutant that grows better at high pressure than at atmospheric pressure (Lauro *et al*, 2008). Since the *E. coli* homologues have opposing effects on initiation of DNA replication and the *P. profundum* SS9 mini-Tn*10* mutants have opposite phenotypes with respect to high pressure, this suggests that balancing proteins that enhance or inhibit DNA replication is vital for the ability of *P. profundum* SS9 to grow under a range of pressures. The role of the SeqA homologue in the pressure- and cold-adapted growth of *P. profundum* SS9 will be investigated in the following chapter.

Chapter 4

P. profundum SeqA and cold adaptation

FL28 is a *P. profundum* SS9 mutant with a mini-Tn*10* insertion in *pbpra1039*, which encodes a putative homologue of *E. coli* SeqA (55% protein identity). In *E. coli*, SeqA negatively regulates DNA replication while DiaA enhances replication, but both are required for timely initiation of replication (Messer, 2002; Ishida *et al*, 2004). Interestingly, FL28 was also isolated from a high pressure screen but was characterised as "pressure-enhanced", since it grows better at high pressure than at atmospheric pressure (Lauro *et al*, 2008). Since the *E. coli* homologues have opposing effects on the initiation of DNA replication and the *P. profundum* SS9 mini-Tn*10* mutants have opposite phenotypes when grown at different pressures, comparing the two mutants may provide valuable insights on the role of DNA replication proteins in adaptation to different pressures.

4.1. Physiological characterisation of *P. profundum* FL28

FL28 was characterised as pressure-enhanced due its ability to grow significantly better at 45MPa than at 0.1MPa (Lauro *et al*, 2008). This was done by calculating a pressure sensitivity ratio (Section 3.1) where the optical density at 45MPa was significantly higher than that at 0.1MPa for a defined time point (Lauro *et al*, 2008). These findings were expanded by assessing the growth on solid and liquid media and the morphology of FL28 at 28MPa, the optimal pressure for the growth of *P. profundum* SS9.

4.1.1. FL28 has a severe growth defect in liquid culture

In order to asses the growth of the FL28 mutant in liquid medium over a range of pressures and temperatures, growth curves were constructed by comparing the OD_{600} of SS9R and FL28 over a time course under a combination of 0.1 and 28MPa and 15 and 4°C. Consistent with previous observations (Lauro *et al*, 2008) FL28 was severely affected in growth at 0.1MPa and 15°C compared to SS9R (Figure 4-1 A). Even though growth of the mutant was also inhibited at 28MPa and 15°C compared to the parent strain (Figure 4-1 B), the growth rate of FL28 at 28MPa was higher than at 0.1MPa; this shows that the mutant grows significantly better at 28MPa, which ties in with the pressure-enhanced phenotype previously described at 45MPa (Lauro *et al*, 2008). The growth of FL28 was also severely inhibited compared to SS9R at 4°C at both 0.1 and 28MPa (Figure 4-1 C & D respectively), suggesting that Pbpra1039 plays a key role in the optimal growth of *P. profundum* SS9 at cold temperatures. Taken together these data demonstrate that FL28 shows pressure-enhanced growth at 28MPa compared to 0.1MPa at 15°C and that the growth inhibition of the mutant is exacerbated at 4°C.

4.1.2. FL28 has a generalised growth defect on solid medium but shows improved growth at high pressure

The growth curves clearly showed that FL28 had a growth defect under all tested conditions in liquid medium. FL23 had a defect in both liquid and solid media, which prompted the possibility that the growth defect of FL28 also extended to its colony forming ability on agar. To compare the growth on solid medium of FL28 to SS9R, cultures were serially diluted, spotted on Marine agar plates and incubated at

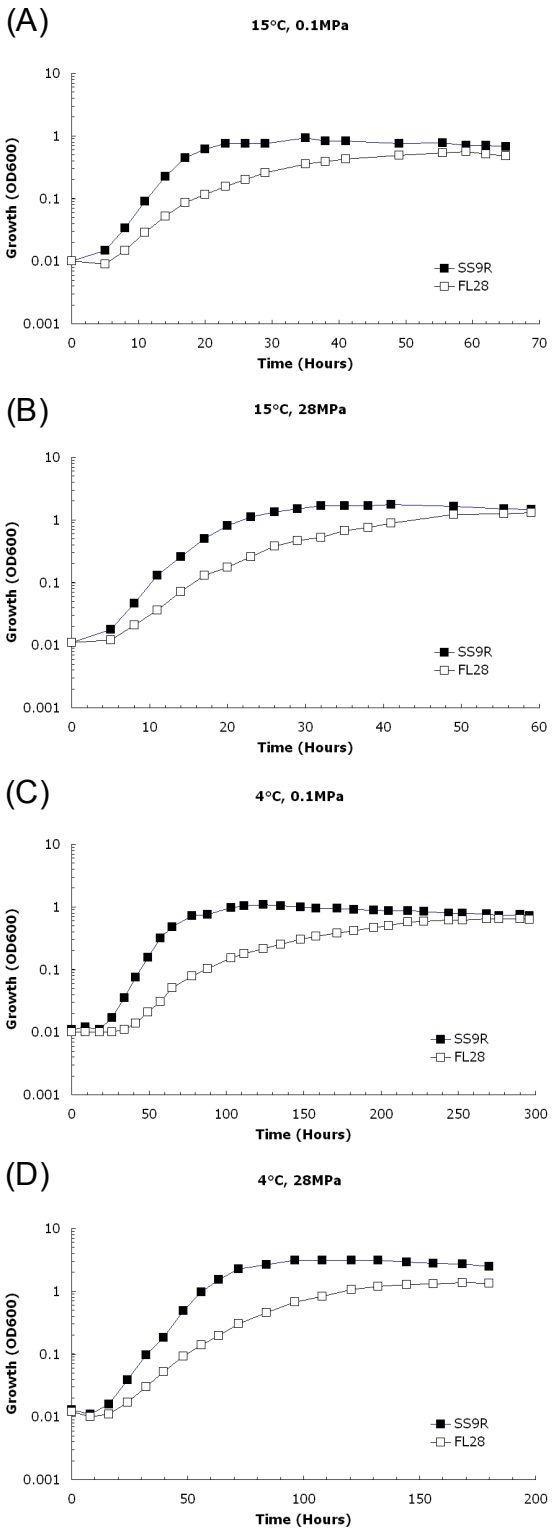


Figure 4-1. Growth of SS9R and FL28 in liquid medium assessed by OD_{600}. Strains were pre-grown at 0.1MPa and 15°C for 48 hours, diluted 1:50 and grown under the same conditions until late exponential phase. They were then diluted to an OD_{600} of 0.01 and grown under the indicated conditions. At each time point growth was assessed by measuring OD_{600}.

0.1MPa and either 15, 9 or 4°C for 96 hours. SS9R was able to grow to a lower serial dilution than FL28 under all three temperatures tested (Figure 4-2), this shows that this mutant is inhibited in its ability to grow on a solid surface relative to the parent.

Since the high pressure-sensitive defect of FL23 was more evident on agar than in liquid medium at 28MPa and 15°C it was thought that this would also be the case for FL28. To test this the growth of FL28 was assessed using the low gelling temperature agar overlay method at 15°C. At 0.1MPa, the growth of FL28 was very poor even on the 10^0 dilution, whereas SS9R grew on the 10^{-1} dilution (Figure 4-3 A). Both parent and mutant strain showed improved growth at 28MPa compared to 0.1MPa, although the growth of FL28 was reduced compared to that of SS9R (Figure 4-3 B). This phenotype was in stark contrast to FL23, which grew to the same dilution at both 0.1 and 28MPa (Figure 3-4). At 4°C FL28 does not seem to grow at all at 0.1MPa, even at the 10^0 dilution, whereas this mutant improved significantly at 28MPa and grew on the 10^{-2} dilution (Figure 4-3 C & D respectively). Taken together these data suggest that FL28 grows worse than the parent on solid medium but that, unlike FL23, growth is improved at high pressure compared to atmospheric pressure.

4.1.3. FL28 cells filament and have abnormal morphologies

In order to determine if the formation of abnormal cellular morphologies is responsible for the growth defects observed with FL28, the cells were examined by phase contrast microscopy at 15°C, 0.1 and 28MPa, and compared to SS9R under the same conditions. Some of the SS9R cells are filamentous at 0.1MPa (Figure 4-4 A) as previously observed (Figure 3-6), but FL28 formed much longer filaments at

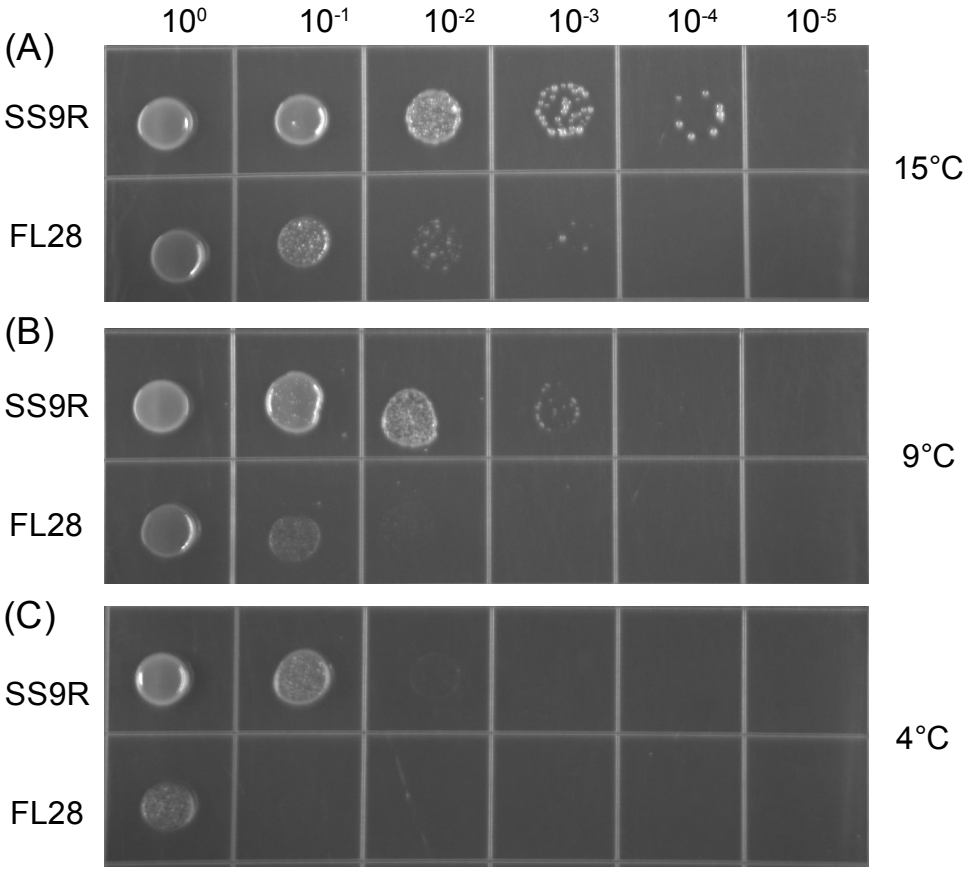


Figure 4-2. Growth of SS9R and FL28 on Marine agar at atmospheric pressure. Liquid cultures were grown to early stationary phase in Marine broth, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted on Marine agar (supplemented with 100mM HEPES and 20mM glucose). Plates were incubated at 0.1MPa at the respective temperatures for 96h. Experiment was reproduced with similar results.

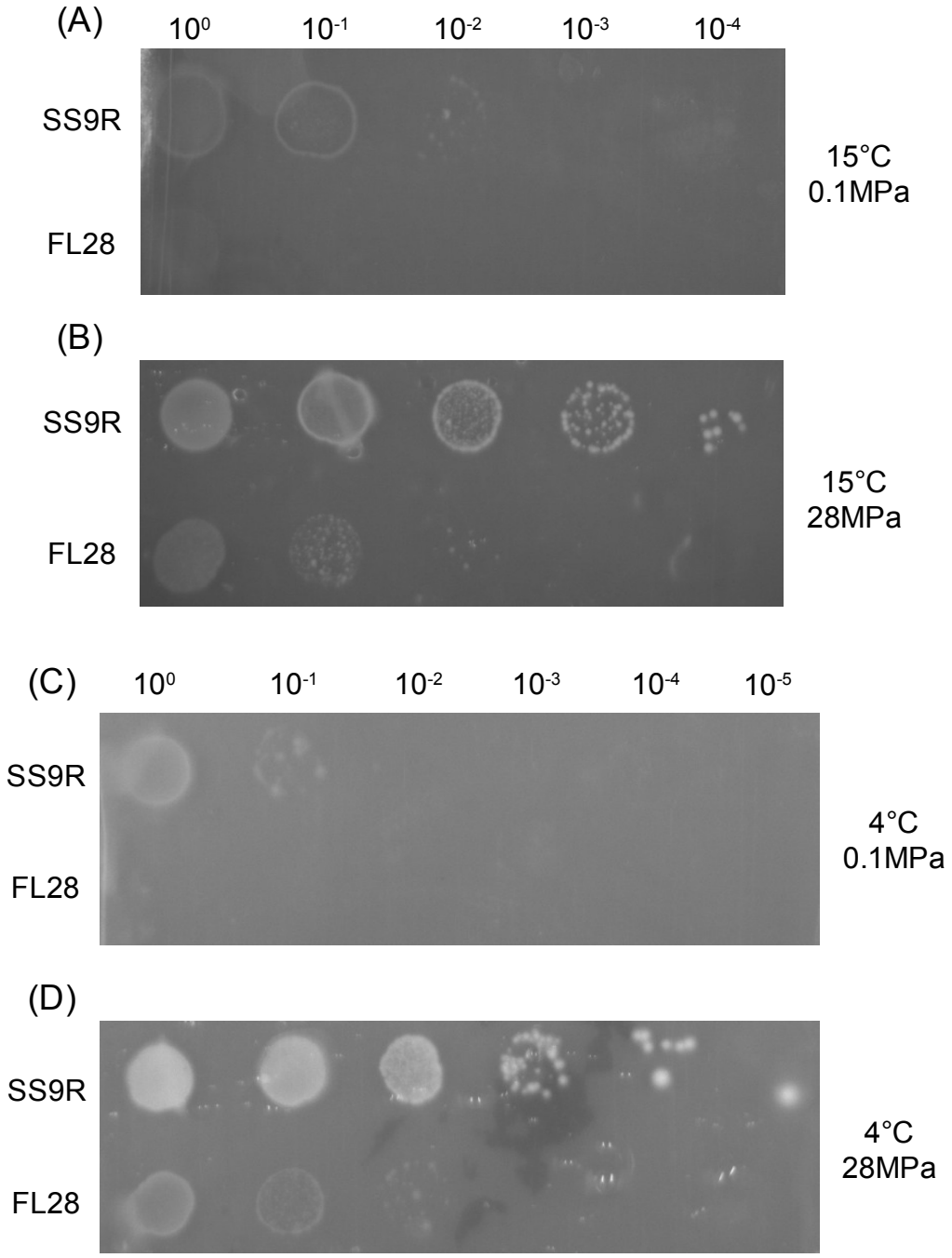


Figure 4-3. Growth of SS9R and FL28 under pressure. Liquid cultures were grown to early stationary phase in Marine broth, diluted to an OD_{600} of 0.8, then serially diluted (10^{-1}-10^{-5}) and spotted on 2.5% LTG Marine agar (supplemented with 100mM HEPES and 20mM glucose). The plates were incubated at 15°C and 0.1MPa for 2 hours, then overlaid with 2.5% LTG Marine agar. Plates were incubated at indicated conditions for 96h (A & B) or 15 days (C & D). Experiment was reproduced with similar results.

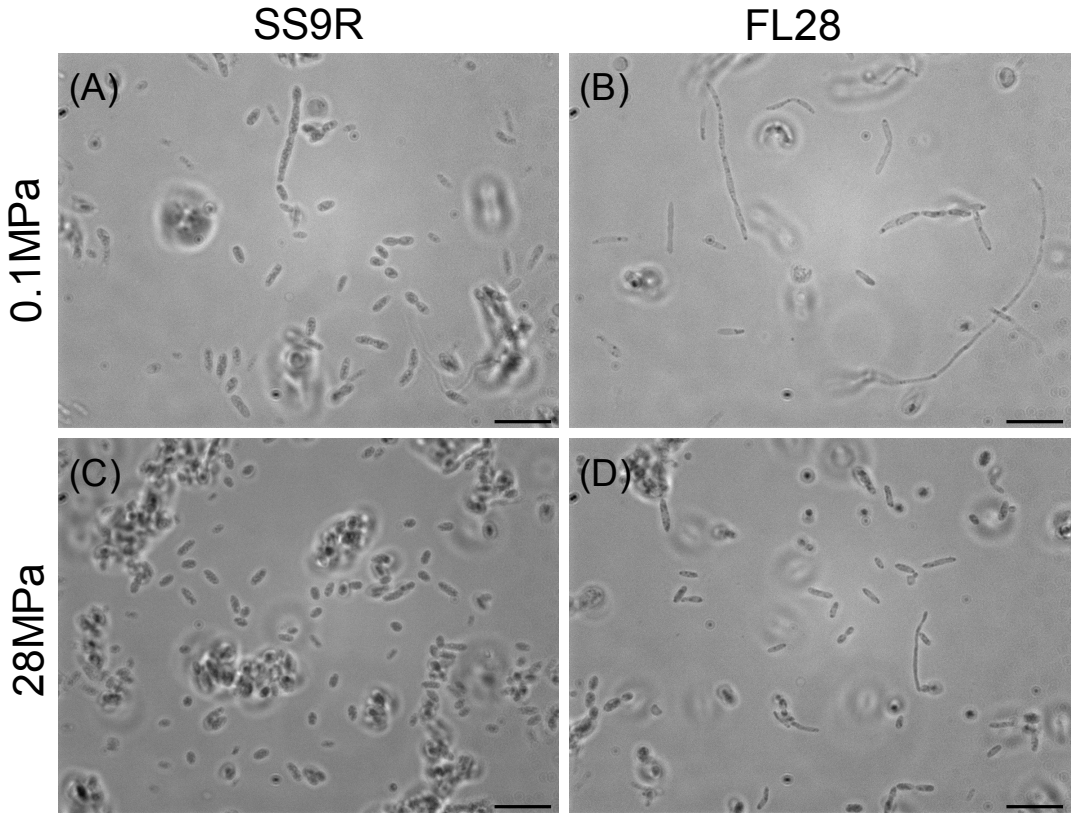


Figure 4-4. Cell morphology of FL28. Liquid cultures were grown to early stationary phase in Marine broth at the indicated pressure, and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. Scale bars represent 10µm. Experiment was reproduced with similar results.

0.1MPa (Figure 4-4 B). Whereas none of the SS9R cells filament at 28MPa (Figure 4-4 C), FL28 formed some long rods and short filaments but not long filaments (Figure 4-4 D). This suggests that loss of Pbpra1039 disrupts cell division in FL28 and that the function of the protein is more critical at atmospheric pressure than high pressure, which ties in with the previously observed pressure-enhanced phenotype of the FL28 mutant at 45MPa.

4.2. Functional characterisation of *P. profundum* Pbpra1039

The FL28 mutant is thought to be lacking the Pbpra1039 protein. Pbpra1039 is annotated as a putative homologue of the inhibitor of DNA replication SeqA and shares 55% amino acid identity with *E. coli* SeqA (Section 1.4.1). The approach used to confirm that Pbpra1039 and SeqA share the same function was to express both proteins in the *E. coli* and *P. profundum* SS9 mutants and check for the ability of the protein from one organism to cross-complement the mutation in the other organism.

4.2.1. Cloning of the pbpra1039 and seqA genes

Once again pFL190 (Lauro *et al*, 2005) was chosen for cloning, since it is the only vector with inducible expression (due to the P_{BAD} promoter) that can replicate in *P. profundum* SS9, and due to the success encountered with both *pbpra3229* and *diaA* complementation. The entire open reading frames of the *E. coli seqA* and *P. profundum* SS9 *pbpra1039* genes, along with their respective ribosome binding sites, were amplified by PCR and cloned into pFL190 to generate the pEC*seqA* and p*pbpra1039* plasmids respectively.

4.2.2. Pbpra1039 overexpression can rescue the cold-sensitive dnaAcos mutant

To gather preliminary evidence that Pbpra1039 and *E. coli* SeqA (EcSeqA) are homologues the ability of *pbpra1039* to substitute for *seqA* in *E. coli* was investigated. The *E. coli seqA* mutant is known to filament when grown in LB and this results in the formation of smaller colonies on LB agar due to the increased doubling time of the mutant (Lu *et al*, 1994), so this could be used to test for the ability of Pbpra1039 to substitute for SeqA. JW0674 is an *E. coli seqA* deletion mutant available from the Keio collection (Baba *et al*, 2006). In order to maintain the same background for all the *E. coli* DNA replication mutants, the *seqA* deletion was moved into KH5402-1 by P1 transduction to generate strain ZE0674. Cultures of the parent and mutant strains were then serially diluted and spotted on LB agar. Both strains grew to the same serial dilution, although as expected smaller colonies were observed with ZE0674 (Figure 4-5 A). However attempts at using colony size to assess complementation of the *seqA* mutant were unsuccessful as the difference in colony size was not as clear-cut with the presence of streptomycin and arabinose in the medium (Figure 4-5 B).

Rather than working with an *E. coli seqA* mutant, the *dnaAcos* background was again taken advantage of. As mentioned previously (Section 3.2.4), the *E. coli dnaAcos* mutant NA001 does not grow at 30°C but grows normally at 42°C. The study that identified EcSeqA had observed that overexpression of wild-type *seqA* in the *dnaAcos* background inverted this phenotype and made the strain grow at 30°C but not at 42°C (Lu *et al*, 1994). This is likely due to the higher levels of SeqA and subsequently increased inhibition of DNA replication compensating for the

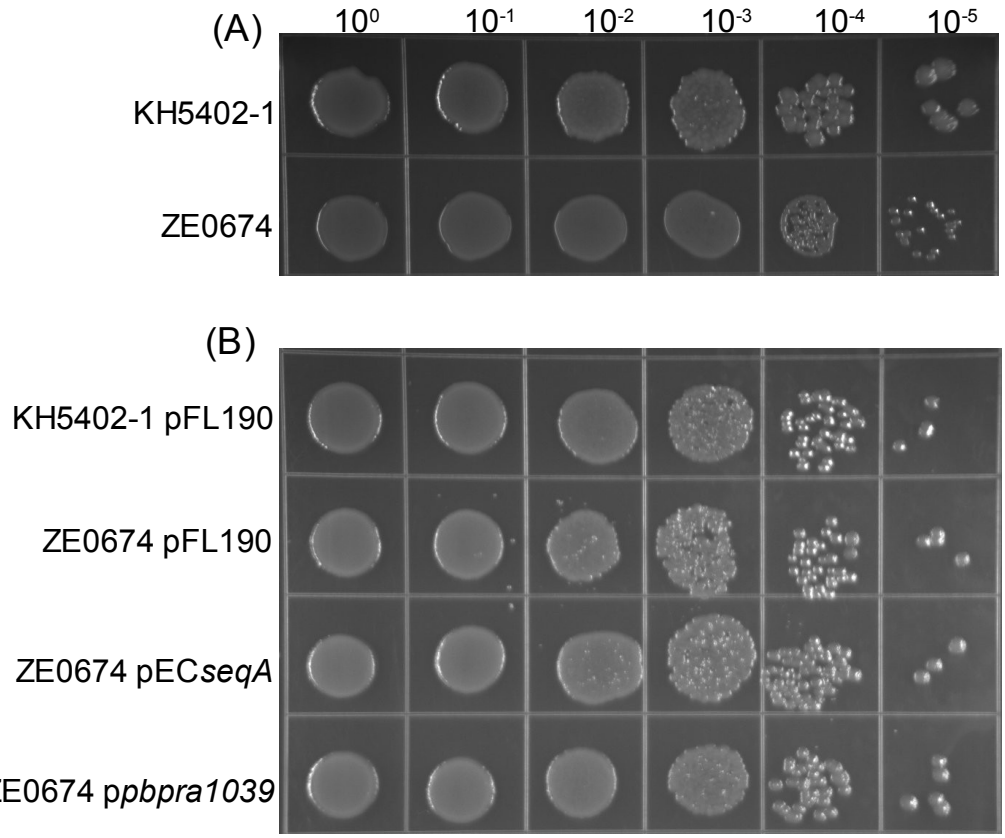


Figure 4-5. Growth of the *E. coli seqA* mutant on agar. The *E. coli* parent (KH5402-1) and *seqA* mutant (ZE0674) were grown overnight in LB broth supplemented with thymine, diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted on LB agar supplemented with thymine (A) or thymine, arabinose and streptomycin (B). Plates were incubated at 37°C overnight. Experiment was reproduced with similar results.

overinitiation associated with *dnaAcos* at 30°C, whereas this increased inhibition leads to a block in DNA replication and subsequent inhibition of growth at 42°C. Therefore if Pbpra1039 is a functional homologue of EcSeqA, overexpression of the former could compensate for overinitiation at 30°C in a similar manner and rescue the cold-sensitivity phenotype. This was tested by transforming pEC*seqA* and p*pbpra1039* into NA001 and assessing the ability of the plasmids to rescue the cold-sensitivity of *E. coli dnaAcos* mutant. Induction of the P_{BAD} promoter on both plasmids with 0.01% (w/v) arabinose resulted in improved growth at 30°C and no change at 42°C (Figure 4-6 A & B respectively), which suggests that Pbpra1039 negatively regulates DNA replication similar to EcSeqA and contributes to timely initiation of replication. Inducing the promoter with 0.2% (w/v) arabinose similarly rescued the cold-sensitive phenotype at 30°C (Figure 4-6 C). However using this higher arabinose concentration at 42°C led to an inhibition of growth when *E. coli seqA* was overexpressed, as expected, but not when *pbpra1039* was overexpressed (Figure 4-6 D) implying that Pbpra1039 is not inhibiting DNA replication to the same extent as EcSeqA at 42°C. Taken together these data show that high levels of Pbpra1039 overexpression is similar to that of EcSeqA at 30°C, however since Pbpra3229 but not EcSeqA supported growth at 42°C this suggests that Pbpra1039 is likely to be a heat-sensitive homologue of SeqA.

4.2.3. Pbpra1039 can partially complement the DNA replication defect of the seqA mutant

The ability of Pbpra1039 and EcSeqA to rescue the cold-sensitive *E. coli dnaAcos* mutant to the same extent at 30°C provided evidence that the proteins were

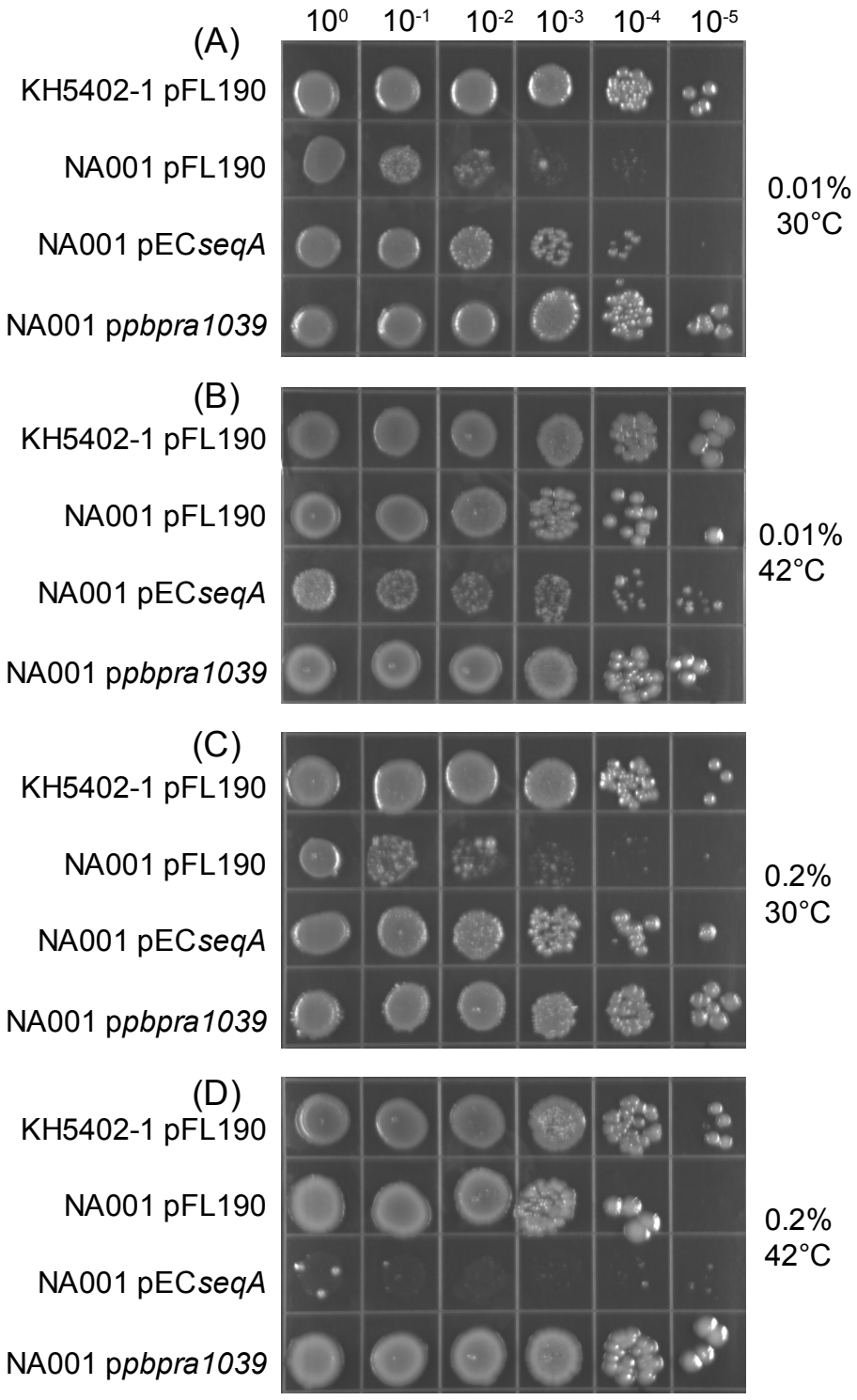


Figure 4-6. Overexpression of Pbpra1039 and SeqA in the *dnaAcos* background. The *E. coli* parent (KH5402-1) and *dnaAcos* mutant (NA001) were grown overnight in LB broth supplemented with thymine (50µg/ml)and streptomycin (100µg/ml), diluted to an OD_{600} of 0.2, then serially diluted (10^{-1}-10^{-5}) and spotted on LB agar supplemented with thymine, streptomycin (100µg/ml) and arabinose (0.01% or 0.2% w/v as indicated). Plates were incubated at 30°C or 42°C overnight. Experiment was reproduced with similar results.

functional homologues and that Pbpra1039 was involved in DNA replication. Further evidence of this could be provided by checking for the ability of wild-type *pbpra1039* to complement the DNA replication defect of the *E. coli seqA* mutant. To this end pFL190, pEC*seqA* and p*pbpra1039* were transformed into ZE0674, the *E. coli seqA* mutant, and DNA replication in the three constructs was analysed by flow cytometry and compared to that of KH5402-1 pFL190, the parent strain carrying the control vector. As in previous experiments, the parent strain showed two major peaks corresponding to 2 and 4 chromosomes as well as a smaller peak for 8 chromosomes (Figure 4-7 A). The mutant carrying the control vector did not show any peaks but instead only noise signal spread across the X axis (Figure 4-7 B). This phenotype was also seen in a previous study in LB and is usually observed when using flow cytometry to analyse cultures with a large proportion of filamentous cells (Lu *et al*, 1994). Expressing wild-type *seqA* in ZE0674 eliminates this phenotype and restores the three wild-type peaks (Figure 4-7 C). Expressing wild-type *pbpra1039* resulted in an intermediate phenotype between mutant and fully complemented, with a significant portion of the background noise still present but stunted forms of the two peaks can still be seen (Figure 4-7 D). This suggests that Pbpra1039 can at least partially complement the DNA replication defect of the *E. coli seqA* mutant, either due to a subtle difference in the function of the two proteins or due to the potential heat sensitivity of Pbpra1039. Taken together these data suggest that Pbpra1039 is a *P. profundum* SS9 SeqA and will be subsequently referred to as PpSeqA.

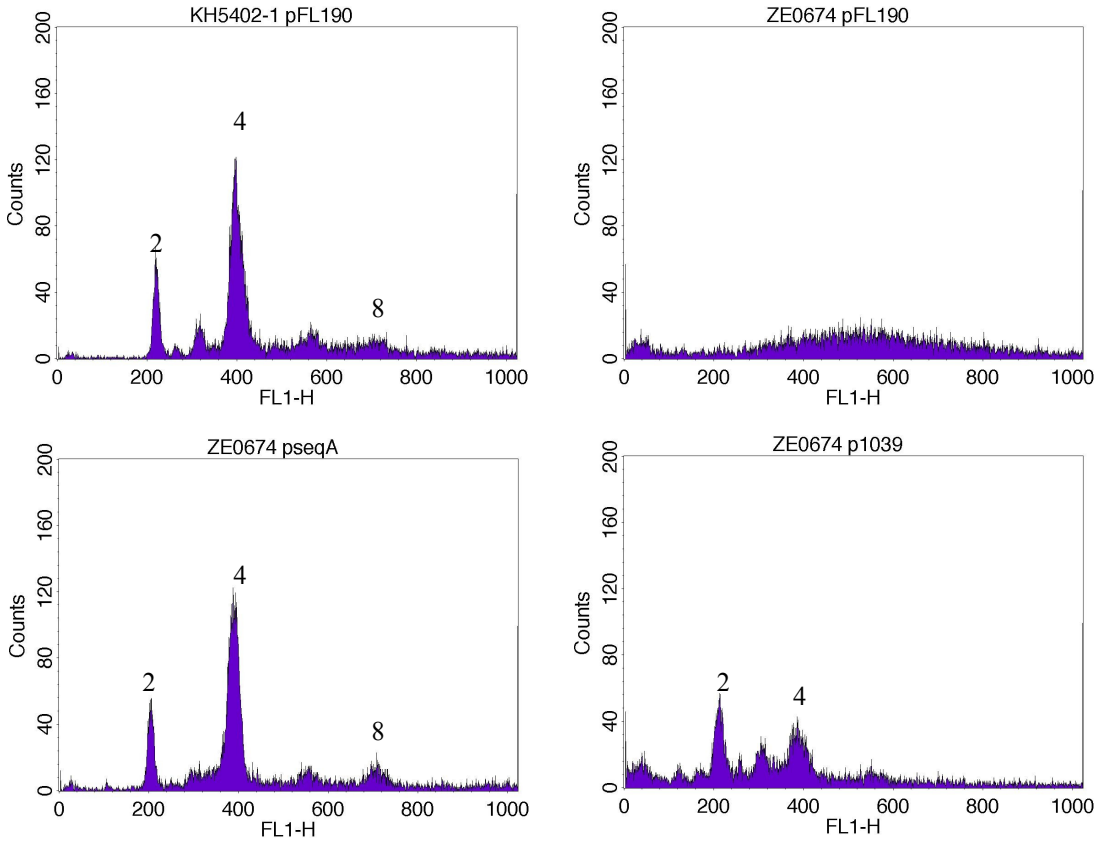


Figure 4-7. Partial complementation of the initiation of DNA replication defect in the *E. coli seqA* mutant by Pbpra1039. The *E. coli* parent (KH5402-1) and *seqA* mutant (ZE0674) were grown at 30°C in LB until mid-exponential phase, treated with rifampicin and cephalexin, fixed in 70% ethanol then the DNA was stained with Sytox Green and analysed by flow cytometry. Chromosome number is indicated above the peaks. Experiment was reproduced with similar results.

4.2.4. SeqA cannot complement the FL28 growth defect at atmospheric or high pressure

Since PpSeqA was at least partially functional in *E. coli*, it was decided to check if EcSeqA could function in *P. profundum* SS9 as well, especially at high pressure. The pFL190, p*pbpra1039* and pEC*seqA* plasmids were conjugated into FL28 and the growth of the strains on solid medium compared to SS9R pFL190 was assessed at atmospheric and high pressure using the low gelling temperature agar overlay method. At 0.1MPa and 15°C, the growth of FL28 pFL190 was reduced compared to that of SS9R pFL190, whereas FL28 p*pbpra1039* grew to the same dilution as the parent (Figure 4-8 A). This shows that the mutation in FL28 is fully complemented by introducing the wild-type *pbpra1039* gene and confirms that the mutant phenotype is solely due to the transposon insertion. However, FL28 p*pbpra1039* did not even grow on the undiluted spot at 28MPa and 15°C (Figure 4-8 B), raising the possibility that maintaining proper control over SeqA expression in *P. profundum* SS9 is much more critical at high pressure. Interestingly FL28 pEC*seqA* failed to complement the growth defect under both pressures, which suggests that EcSeqA is not functional in *P. profundum* SS9 under the investigated conditions.

4.2.5. PpSeqA is a cold-adapted protein

Complementation of the DNA replication defect in the *E. coli seqA* mutant by *pbpra1039* suggested that PpSeqA and EcSeqA were functional homologues. However EcSeqA was unable to complement the FL28 growth defect on solid medium at 15°C. Additionally, overexpression of PpSeqA in the *E. coli dnaAcos* background rescued the cold-sensitivity at 30°C but was not lethal at 42°C whereas

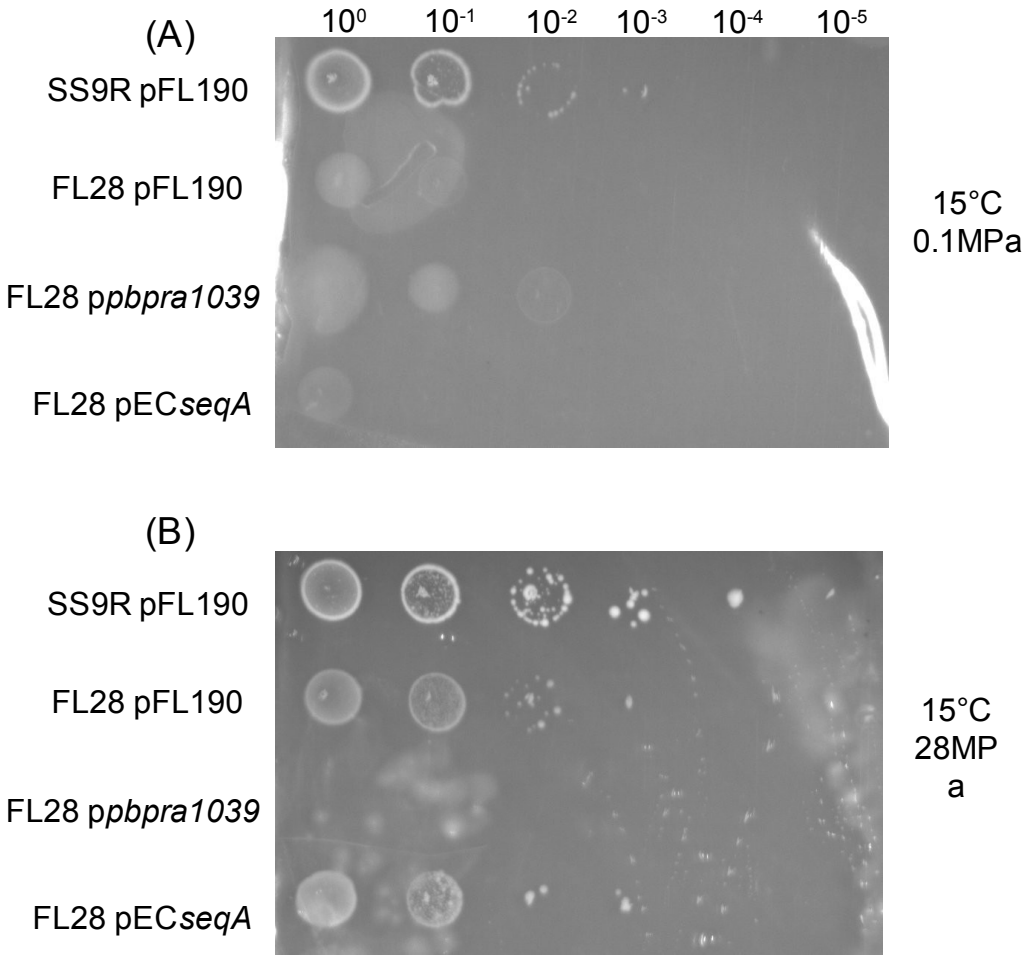


Figure 4-8. Complementation of *P. profundum* FL28 by Pbpra1039. Strains were pre-grown in Marine broth, serially diluted and spotted on LTG Marine agarsupplemented with arabinose (0.01% w/v). The plates were overlaid with LTG Marine agar and incubated at 15°C at the indicated pressures for 96 hours. Experiment was reproduced with similar results.

EcSeqA overexpression was. This fluctuation in phenotype complementation depending on the temperature suggests that temperature may interfere with the function or stability of the SeqA protein in *E. coli* and *P. profundum* SS9. This could not be tested in *P. profundum* SS9 due to its more restricted temperature range. The *E. coli seqA* mutant is known to filament in LB (Lu *et al*, 1994) therefore ZE0674 was chosen to assess the ability of the two proteins to complement the filamentation phenotype at low and high temperatures. The parent with control vector grew as rod-shaped cells at 37°C and 15°C (Figure 4-9 A & B respectively) whereas the mutant with control vector filamented at both temperatures (Figure 4-9 C & D). Introducing pEC*seqA* into the mutant resulted in rod-shaped cells at 37°C but filaments were still present at 15°C (Figure 4-9 E & F), suggesting that pEC*seqA* can complement the mutant phenotype at high but not low temperatures. Conversely, introducing p*pbpra1039* did not complement the filamentation at 37°C but fully complemented the phenotype at 15°C, resulting exclusively in rod-shaped cells (Figure 4-9 G & H). This suggests that PpSeqA can complement the filamentation of the *E. coli seqA* mutant at low temperatures but not at high temperatures. Taken together these data argue that PpSeqA is a cold-adapted *P. profundum* SS9 homologue of SeqA.

4.3. Discussion

The *P. profundum* FL28 mutant has a transposon insertion in the putative *seqA* homologue *pbpra1039*, and was defined as “pressure-enhanced” in liquid medium at 45MPa. This study expanded the mutant's physiological characteristics and determined that Pbpra1039 is likely to be a cold-adapted homologue of SeqA.

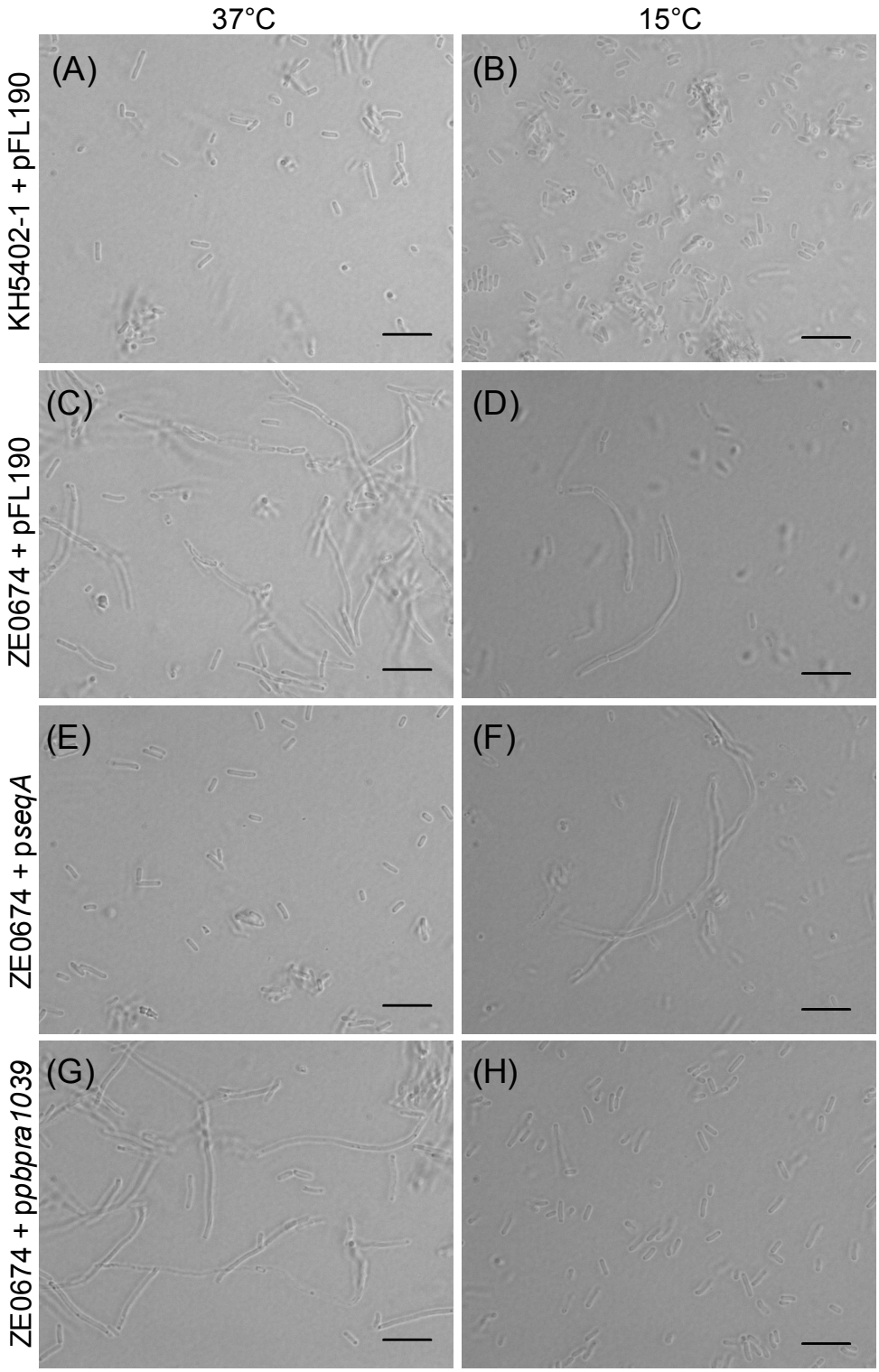


Figure 4-9. Complementation of the *E. coli seqA* mutant at 37 and 15°C. The *E. coli* parent (KH5402-1) and *seqA* mutant (ZE0674) were grown to early stationary phase in LB broth at the indicated temperature, and 10µl of culture was spotted on a glass slide coated with poly-L-lysine to immobilise the cells. Images were taken with a 100x oil immersion phase contrast lens. Scale bars represent 10µm. Experiment was reproduced with similar results.

4.3.1. Growth phenotype of FL28

FL28 was defined as pressure-enhanced because its doubling time at 0.1MPa was increased compared to 45MPa at 17°C (Lauro *et al*, 2008). When the growth of the mutant was assessed at 0.1 and 28MPa this increase in doubling time at atmospheric pressure was still observed, even though the mutant had an increased lag phase and increased doubling time compared to the parent under all conditions observed. Under all conditions observed the growth of FL28 in liquid medium is more severely inhibited than FL23, which suggests that the absence of the SeqA homologue is more disruptive than that of the DiaA homologue. Observation of the cellular morphology of the *pbpra1039* mutant confirmed the pressure-enhanced phenotype, as FL28 formed long rods and short filaments at 28MPa but much longer filaments at 0.1MPa. This suggested that loss of Pbpra1039 causes these abnormal morphologies at atmospheric pressure, which in turn leads to a significant decrease in growth rate.

4.3.2. Pbpra1039 is a P. profundum homologue of SeqA

Pbpra1039 was annotated as a putative homologue of the inhibitor of DNA replication SeqA and the two proteins shared significant (55%) amino acid identity. Unlike the *E. coli diaA* mutant, which shows no visible growth or morphology defects, the *E. coli seqA* mutant filaments when grown in LB and as a result does not show the characteristic two peaks when its DNA content is analysed by flow cytometry. Introducing *pbpra1039* into the *E. coli seqA* mutant partially restored timely initiation of DNA replication, which suggested that Pbpra1039 is a *P. profundum* homologue of SeqA.

E. coli SeqA has been most extensively studied in DNA replication but is also known to act as a transcription factor at certain promoters (Słomińska *et al*, 2001). A microarray analysis of genes affected by SeqA revealed that expression of a large number of SOS response genes is increased in the *seqA* mutant (Løbner-Olesen *et al*, 2003). The SOS response blocks FtsZ polymerisation and subsequently inhibits septation, leading to cell filamentation (Huisman *et al*, 1984). Therefore the filamentation phenotype observed in the *pbpra1039* and *E. coli seqA* mutants could be due to the induction of the SOS response in these mutants rather than being a direct consequence of disruption in timely initiation of DNA replication.

4.3.3. PpSeqA and EcSeqA are functional over different temperature ranges

The ability of PpSeqA only partially to complement the flow cytometry phenotype of the *E. coli seqA* mutant raised the possibility that, even though PpSeqA and EcSeqA were functional homologues, the function of each protein was more specific to its parent organism. This seemed to be confirmed by EcSeqA not complementing the growth defect on agar of *P. profundum* FL28. However PpSeqA overexpression in *E. coli dnaAcos* mutant rescued the cold-sensitivity of this mutant at 30°C, but unlike EcSeqA was not lethal at 42°C, suggesting that temperature has an effect on the function of PpSeqA and EcSeqA, rather than which organism the proteins are introduced in. This hypothesis was evidenced by the ability of PpSeqA to complement the filamentation of the *E. coli seqA* mutant at 15°C but not 37°C. The body of evidence points towards PpSeqA being a *P. profundum* SS9 cold-adapted protein.

The ability of pEC*seqA* to rescue the filamentation defect in the *E. coli seqA* mutant at 37°C but not 15°C is puzzling, since introducing wild-type *seqA* should complement the mutation regardless of the temperature. SeqA overexpression at high levels is known to delay initiation of DNA replication and cell division and results in cell filamentation (Bach *et al*, 2003) but mutant cells carrying pEC*seqA* were only filamentous at 15°C. This filamentation was observed when different concentrations of arabinose were used to induce protein expression, which suggests that the balance in cellular levels of the EcSeqA protein is more delicate at 15°C than at 37°C, resulting in disruption to initiation of replication when this balance is not maintained at 15°C.

Overproduction of PpSeqA was lethal in *P. profundum* FL28 at 28MPa but properly complemented the mutation at 0.1MPa. It could be argued that PpSeqA is more active at the optimal pressure of the organism and this would lead to increased inhibition of replication at 28MPa. Alternatively, initiation of DNA replication is more sensitive at high pressure and the initiation machinery is more therefore more sensitive to an increase in the number of SeqA molecules at high pressure. Unlike *E. coli*, where overexpression of *seqA* inhibits initiation of DNA replication but doesn't affect cell viability (Lu *et al*, 1994), overproduction of SeqA in *V. cholerae* is lethal (Saint-Dic *et al*, 2008). Since *P. profundum* and *V. cholerae* are closely related and thought to share similar mechanism of DNA replication, overproduction of PpSeqA could be lethal at the optimal pressure for *P. profundum* SS9 as observed by the overlay method. Because of the difficulty in controlling the precise level of SeqA, even using the P_{BAD} promoter, the exact problem is difficult to elucidate.

Chapter 5

Isolating cell envelope polysaccharides from *P. profundum* SS9 and a metagenomic library

This PhD was funded in part through the Leverhulme Trust, whose aim is to develop novel materials. A surface water metagenomic library was constructed to set up screens and selections that could be eventually used to identify DNA fragments from a deep-sea metagenomic library. Additionally, novel polysaccharides isolated from the marine library could still be potentially interesting in the food industry to improve food texture (Dabour *et al*, 2006), as thickening agents (De Vuyst and Degeest, 1999), or in medicine as anti-tumour agents (Zhang *et al*, 2005a). A set of techniques was also developed to identify cell envelope polysaccharides from *P. profundum* SS9.

5.1. Construction of a surface seawater metagenomic library

The metagenomic library from the DNA of marine microorganisms was constructed by Dr Gail Ferguson, who filtered 40L of surface seawater from Woods Hole Passage (Massachusetts, USA) and collected the microorganisms on a 0.1μm filter. The microorganisms were lysed and the DNA was extracted, purified and cloned into a fosmid vector, pCC1FOS (Epicentre). The fosmids were packaged into bacteriophage λ, and then transduced into *Escherichia coli* EPI300 (Figure 5-1 A). The fosmid library contains 44,000 clones, and the packaging steps ensured that each clone carries an insert of around 40kb. The fosmid has two origins of replication

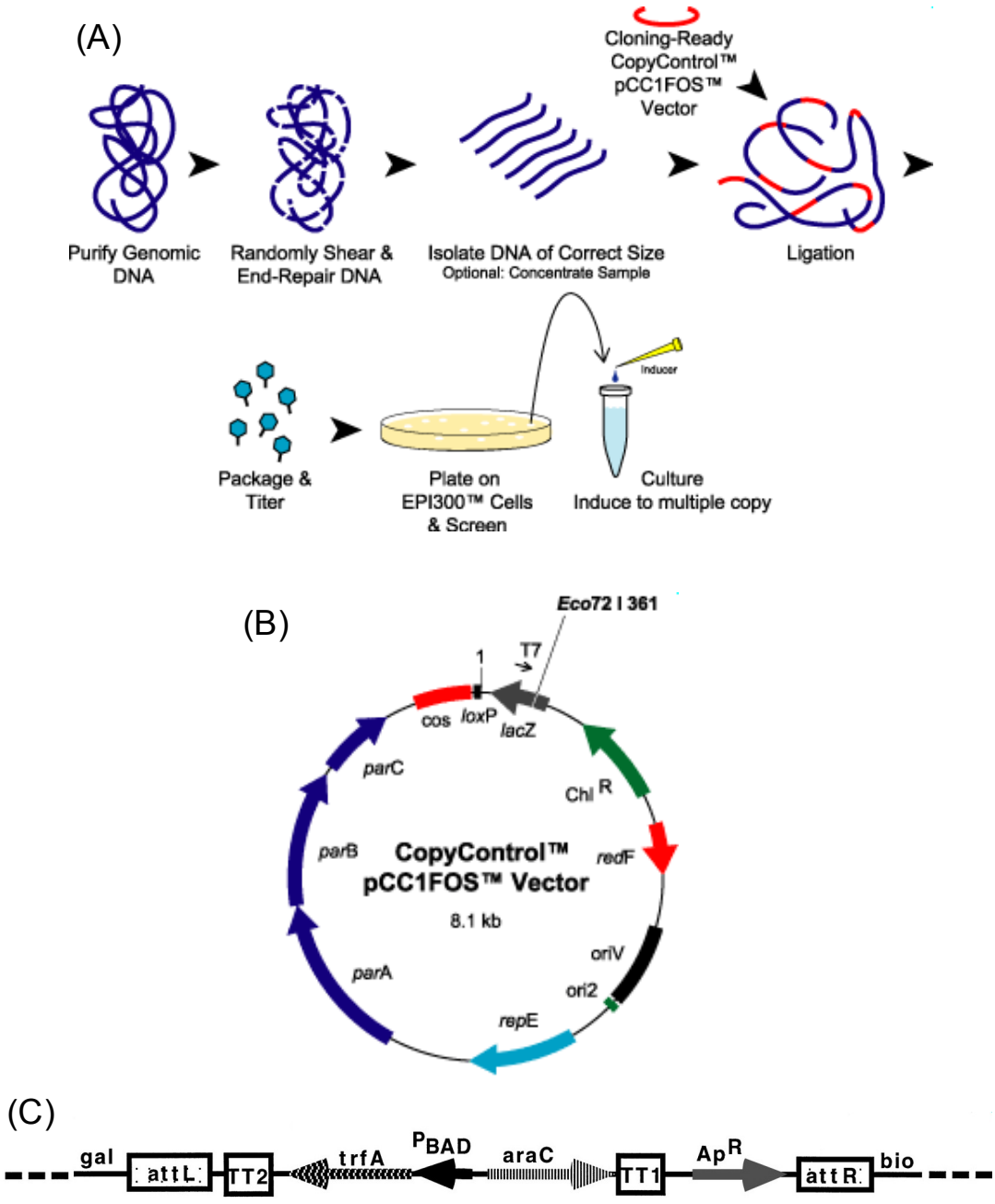


Figure 5-1. Construction of the Woods Hole marine metagenomic library. A: Purified DNA recovered from the marine microorganisms was randomly sheared by pipetting 500 times, then cloned into the pCC1FOS vector. The fosmids were packaged into bacteriophage λ then transduced into *E. coli* EPI300. B: The pCC1FOS fosmid. Shown are the *cos* site required for packaging into bacteriophage λ, the chloramphenicol resistance gene (Chl^R), the *ori2* and *oriV* origins of replication and the maintenance of stability system consisting of *parABC*, *repE* and *redF*. The cloning site is within the *lacZ* gene, which allows for blue/white selection of positive clones on media containing X-Gal. C: Schematic of the *trfA* gene under control of the P_{BAD} promoter in the EPI300 strain (sources: Epicentre; Wild *et al*, 2002)

(Figure 5-1 B). Under normal conditions only *ori2*, the F-plasmid origin of replication, is active and the fosmid is present in single copy in the host cell. The *oriV* origin is activated in the presence of the replication factor TrfA, and this induces the fosmid to high copy number (Kornacki *et al*, 1984). This control system only functions in the EPI300 strain because the *E. coli* genome does not code for TrfA, and EPI300 has been specifically engineered to introduce the *trfA* gene into the chromosome (Figure 5-1 C), under the control of the L-arabinose inducible promoter $P_{ara\text{BAD}}$ (Khlebnikov *et al*, 2002). Therefore, the fosmid copy number can be increased simply by addition of arabinose to the growth medium.

A separate 16S rRNA gene library that could be used to evaluate bacterial diversity in the sample was also constructed from the metagenomic library by Dr Gail Ferguson. The 16S rRNA genes were amplified directly from the environmental DNA by PCR using the 8F and 1492R primer pair, which specifically allows amplifications of the bacterial 16S rRNA genes. The PCR products were cloned into the TOPO vector (Invitrogen) and transformed into *E. coli* TOP10 competent cells (Invitrogen).

5.2. Assessing diversity in the metagenomic library

Since the goal was to identify novel materials in uncultured organisms, it was vital to ensure that microorganisms represented in the metagenomic library were closely related to uncultured microorganisms. The phylogenetic diversity in the metagenomic library was assessed using 16S rRNA genes. Transformants were plated on LB agar, and 96 clones were inoculated. Plasmids were extracted from these clones and the 16S rRNA genes were sequenced using the 8F primer. The

sequences were imported into the ARB software package, aligned and used to construct a phylogenetic tree (Figure 5-2). Microorganisms represented in the metagenomic library seemed to be distributed throughout the bacterial domain, with the majority being located within the various proteobacteria groups (Figure 5-3 A). A closer look at the representation within the γ-proteobacteria showed that the microorganisms were diversely distributed even within specific subgroups of bacteria and many were close relatives of uncultured microorganisms (Figure 5-3 B). Therefore the distribution of bacteria within the library was deemed to be diverse and representative of uncultured marine microorganisms.

5.3. Attempts at selecting for clones with specific resistance phenotypes

Cell envelope modifications are induced in response to both cold temperature (Rilfors *et al*, 1978) and high pressure (Welch and Bartlett, 1996), therefore constructing a deep-sea metagenomic library and identifying DNA fragments that alter the cell envelope composition of an *E. coli* host and confer cold and/or pressure resistance on that host could provide valuable insights into deep-sea adaptations. The surface seawater metagenomic library was used to test methods and techniques that could be eventually applied to a deep-sea metagenomic library and to gain better insights into the mechanisms of cold-adapted growth.

5.3.1. Selecting for metagenomic clones with increased cold resistance

Although cold adaptation is thought to be a complex process, a minimal set of required genes may exist that, if cloned into a mesophile such as *E. coli*, could be

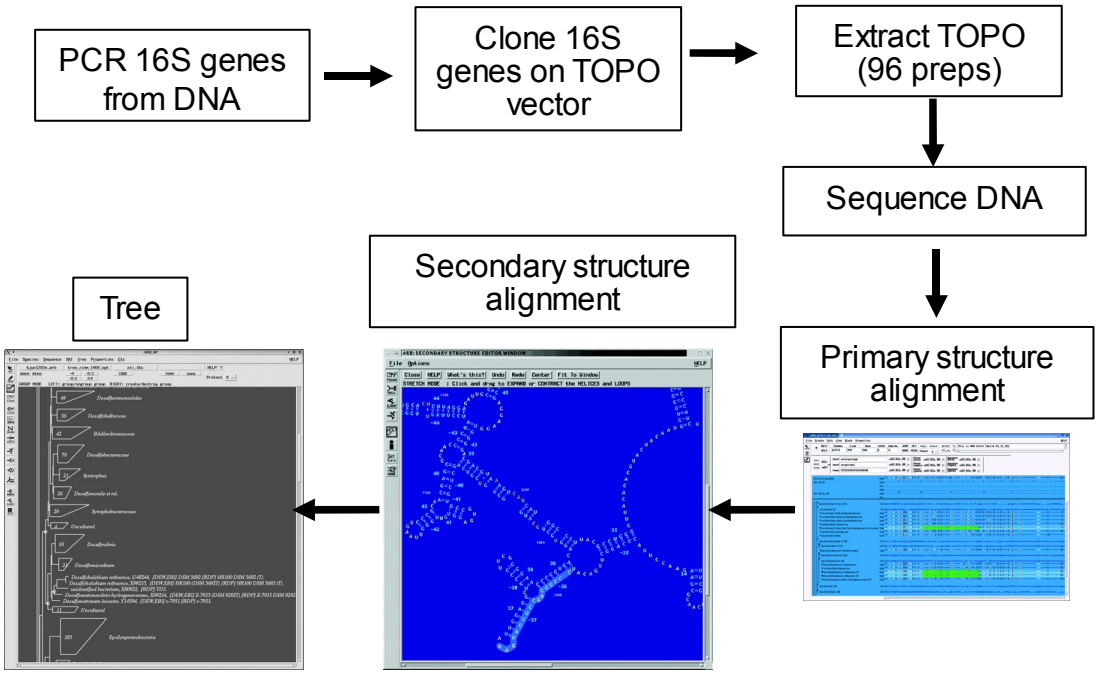
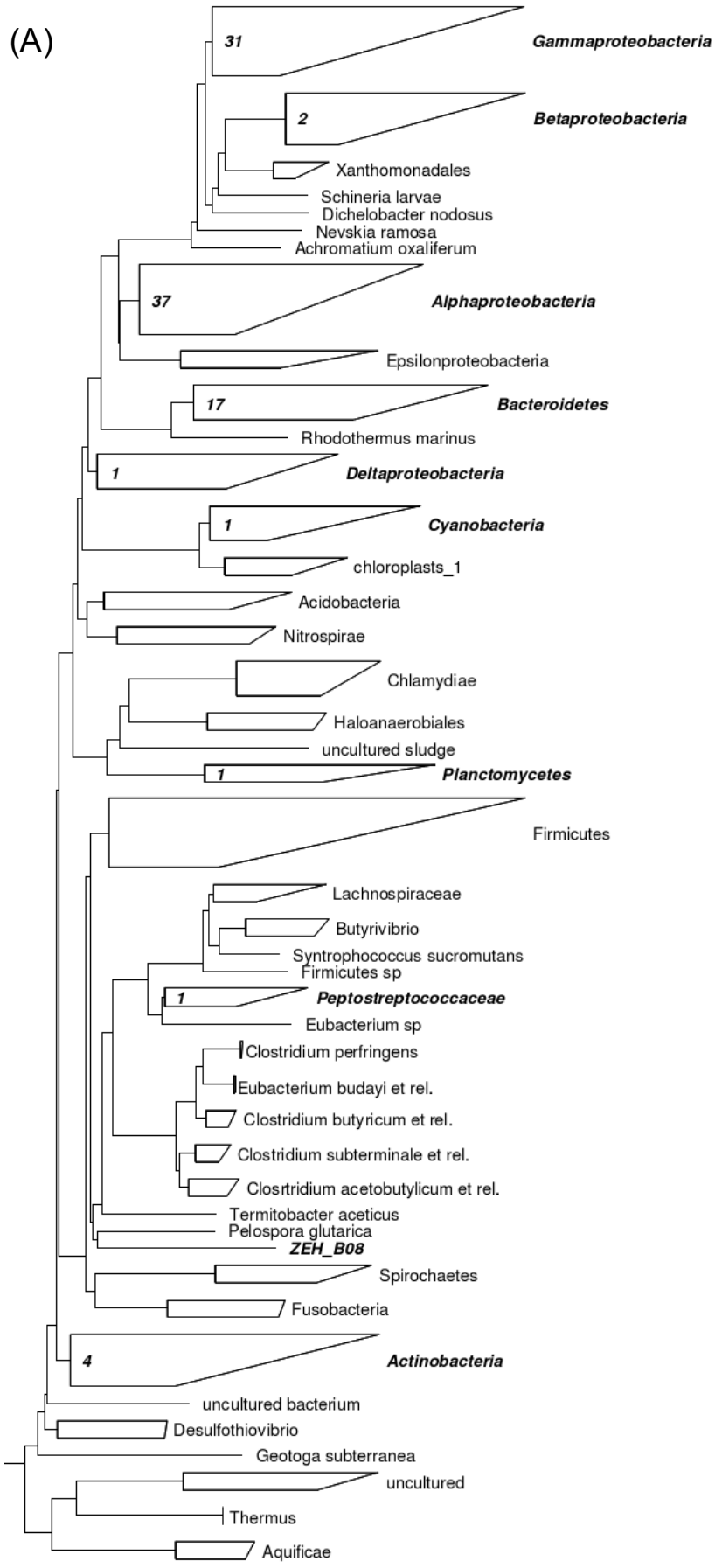


Figure 5-2. 16S rRNA sequence alignment and tree construction using ARB. 16S rRNA genes were amplified using the 8F/1492R primer pair, cloned into the TOPO vector and transformed into TOP10 cells to create the 16S library. 96 clones were then extracted, sequenced and aligned in ARB. The secondary structure was manually refined and used to generate a phylogenetic tree.


(A)
31
Gammaproteobacteria
2
Betaproteobacteria
Xanthomonadales
Schineria larvae
Dichelobacter nodosus
Nevskia ramosa
Achromatium oxaliferum
37
Alphaproteobacteria
Epsilonproteobacteria
17
Bacteroidetes
Rhodothermus marinus
1
Deltaproteobacteria
1
Cyanobacteria
chloroplasts_1
Acidobacteria
Nitrospirae
Chlamydiae
Haloanaerobiales
uncultured sludge
1
Planctomycetes
Firmicutes
Lachnospiraceae
Butyrivibrio
Syntrophococcus sucromutans
Firmicutes sp
1
Peptostreptococcaceae
Eubacterium sp
Clostridium perfringens
Eubacterium budayi et rel.
Clostridium butyricum et rel.
Clostridium subterminale et rel.
Closrtridium acetobutylicum et rel.
Termitobacter aceticus
Pelospora glutarica
ZEH_B08
Spirochaetes
Fusobacteria
4
Actinobacteria
uncultured bacterium
Desulfothiovibrio
Geotoga subterranea
uncultured
Thermus
Aquificae

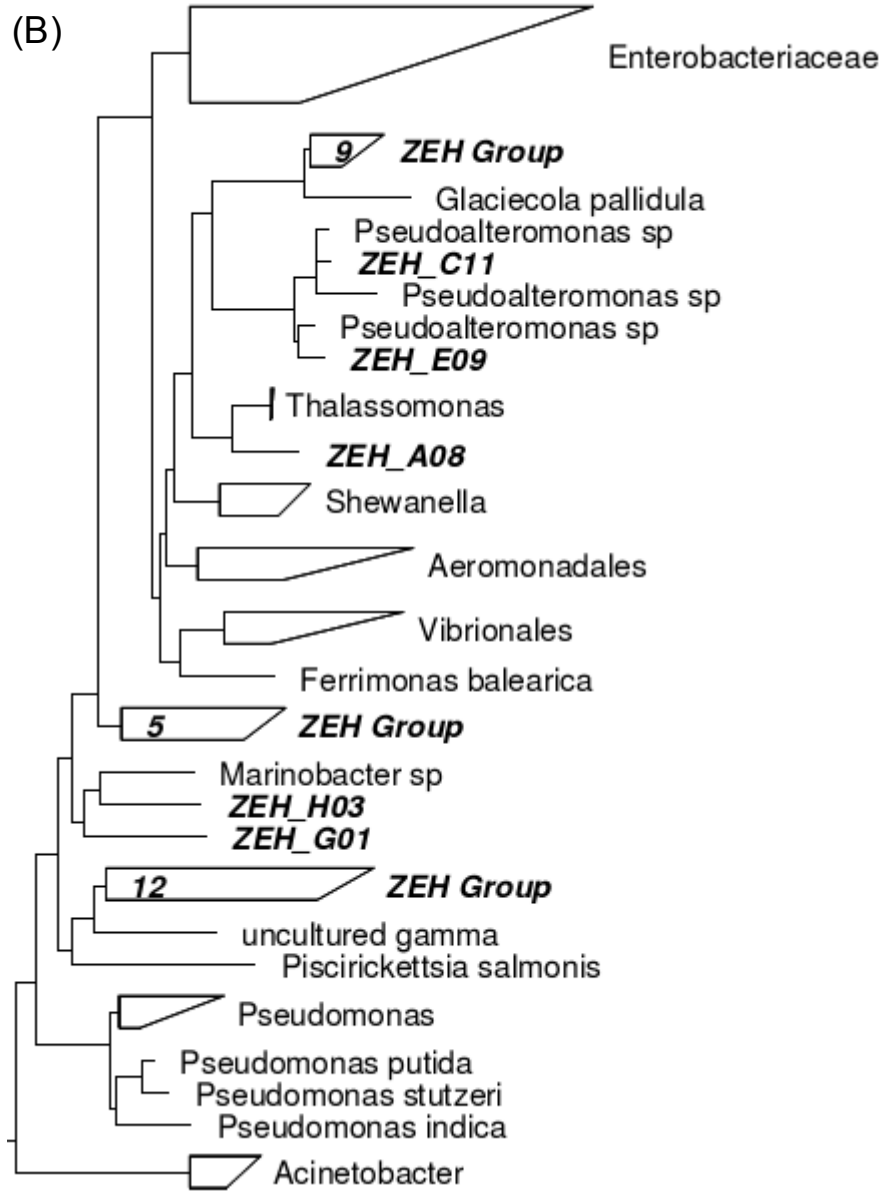


Figure 5-3. Microbial diversity in the metagenomic library. A (previous page): 16S rRNA genes were sequenced and aligned using ARB. The phylogenetic tree shows the bacterial groups within which microorganisms represented in the metagenomic library are distributed. B: Subtree showing the microbial distribution from the metagenomic library within the γ-proteobacteria. The numbers represent how many clones are within a group and the group names are in italic bold.

sufficient to increase resistance to cold and support growth at temperatures where the mesophile naturally cannot survive. In one study, the Cpn60/10 chaperones from the psychrophile *Oleispira antarctica* were expressed in an *E. coli* K-12 derivative, and this was enough to increase the resistance of the *E. coli* strain and allow it to grow at temperatures as low as 4°C (Ferrer *et al*, 2003).

Although the metagenomic library was constructed from microorganisms isolated from surface seawater, it is possible that some of them originated from lower depths and moved to the surface through currents. Since the average temperature of the sea is 3°C (Bruun, 1957), these microorganisms would be cold adapted. In order to identify metagenomic clones that could confer increased cold resistance upon *E. coli*, the growth of EPI300 was assessed at cold temperatures. EPI300 can grow at 15°C but not at 9°C and 4°C (Figure 5-4). Metagenomic DNA that confers cold resistance can therefore be isolated by selecting for clones that can grow at these temperatures. To isolate such clones, $1.5x10^6$ cells were spread on LB agar and incubated at either 9°C or 4°C. After 10 days, seven colonies were visible at 9°C and one colony was visible at 4°C. These were streaked on LB agar at 37°C overnight, and single colonies were restreaked on LB agar and incubated at 9°C. However none of the clones grew and further attempts at getting them to grow at temperatures below 10°C were unsuccessful. Since the cold resistance phenotype was not stable, these clones were not pursued further.

5.3.2. Selecting for metagenomic clones with increased resistance to detergents

Polysaccharide components of the outer membrane are known to confer

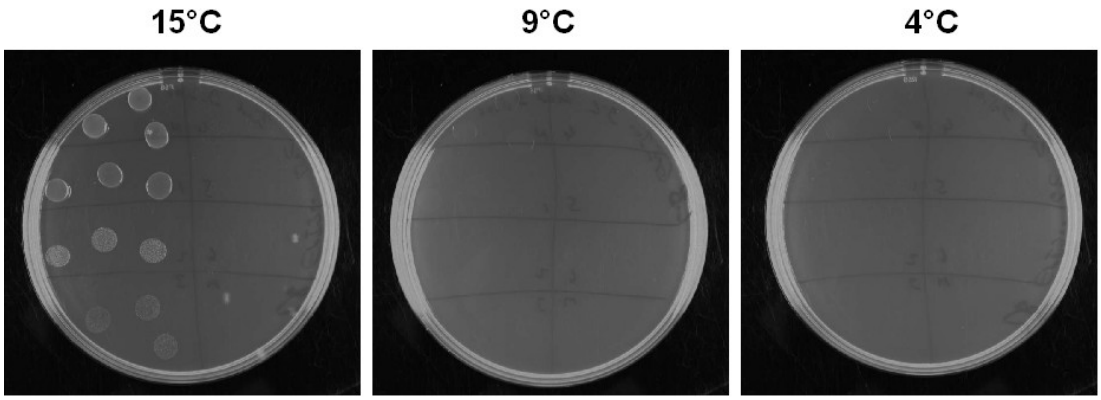


Figure 5-4. EPI300 growth at cold temperatures. An EPI300 liquid culture was grown overnight at 37°C, diluted to an OD_{600} of 0.2, serially diluted (10^{-1}-10^{-6}) and then spotted in triplicate on LB agar containing chloramphenicol (12.5µg/ml) and arabinose (0.01% w/v). Plates were incubated at the indicated temperature for 10 days.

resistance to detergents in gram-negative bacteria (Nixdorff *et al*, 1977). Therefore, it is possible to identify novel cell envelope components by selecting for clones with altered resistance to detergents such as sodium deoxycholate (DOC) and sodium dodecyl sulphate (SDS). To facilitate this, a concentration of detergents at which EPI300 was sensitive was first determined, as this would allow identification of clones conferring increased resistance to a detergent by positive selection. However, neither detergent on its own prevented growth of EPI300, although growth was slower in the presence of DOC, as seen by formation of smaller colonies (Figure 5-5 B & E). Divalent cations are known to stabilise the outer membrane by cross-linking polysaccharides and chelating these cations with EDTA has been shown to sensitise cells to the detergent Triton X-100 (Schnaitman, 1971). Therefore, EDTA was also added to the agar to increase the sensitivity of EPI300 to SDS and DOC. Low EDTA concentrations had a slight inhibitory effect on EPI300 growth (Figure 5-5 C & F), but these same concentrations in combination with either DOC or SDS prevented growth on LB agar (Figure 5-5 D & G), providing a means to identify genes involved in resistance to detergents using positive selection.

After determining the exact conditions at which EPI300 was sensitive to detergents, library clones with increased resistance to these detergents were selected by spreading $1.5x10^6$ cells on LB agar plates containing either SDS and EDTA (0.2% w/v and 3mM, respectively), or DOC and EDTA (20mM and 2mM, respectively), in the presence of arabinose. No DOC-EDTA-resistant clones were isolated in this selection, but three SDS-EDTA-resistant clones (termed SE1, SE2 and SE3) were isolated and were confirmed to be resistant to SDS-EDTA (Figure 5-6 A & B), even in the absence of arabinose (Figure 5-6 C & D). To confirm that the resistance was

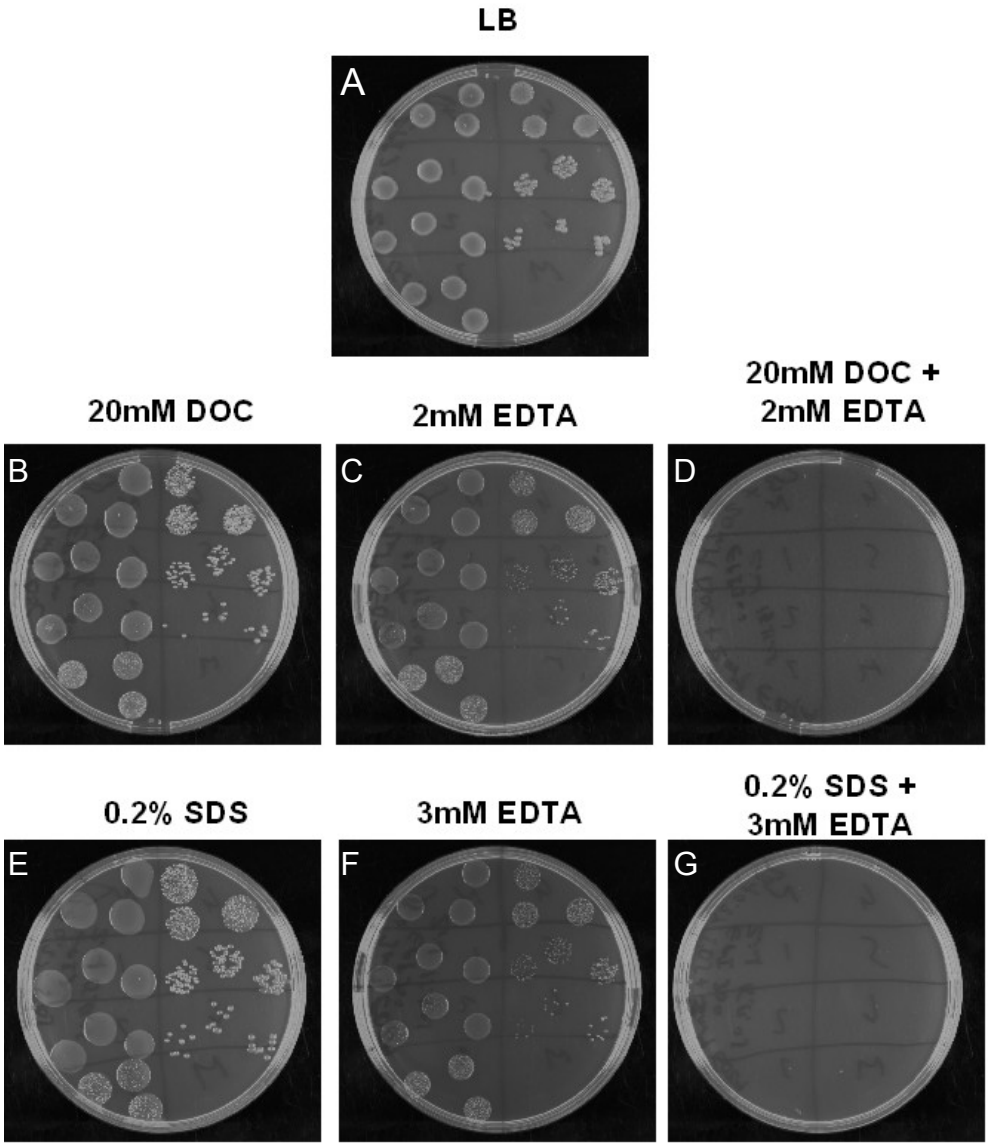


Figure 5-5. Effect of EDTA, DOC and SDS on growth of *E. coli* EPI300. An EPI300 liquid culture was grown overnight, diluted to an OD_{600} of 0.2, serially diluted (10^{-1}-10^{-6}), then spotted in triplicate on LB agar containing SDS (0.2% w/v), DOC (20mM) or EDTA (2mM or 3mM) as indicated. Plates were incubated at 37°C overnight.

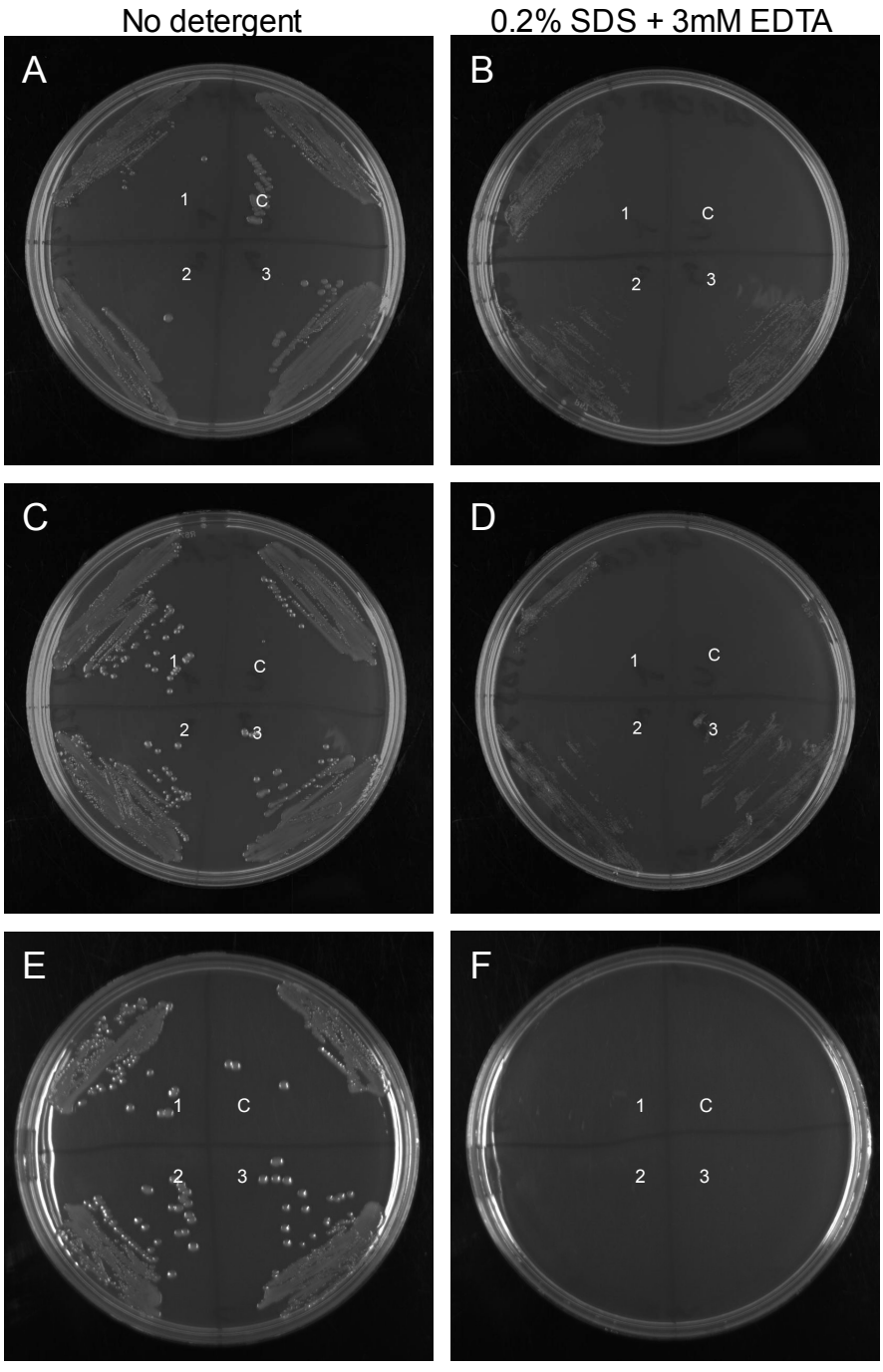


Figure 5-6. Selecting for resistance to detergents from the metagenomic library. SE1, SE2 and SE3 were streaked alongside a control C, which carries the fosmid with no metagenomic DNA, on LB agar with chloramphenicol (A & C) and LB agar with chloramphenicol, SDS (0.2% w/v) and EDTA (3mM) (B & D). After extracting the fosmid and transforming into a fresh EPI300 background, the subclones were streaked as above on LB agar with no detergent (E) or with SDS and EDTA (F). Plates A, B, E and F contained arabinose (0.01% w/v). Plates were incubated at 37°C for 40 hours.

conferred by the metagenomic DNA and not random chromosomal mutations, the fosmid was extracted from the SE clones and transformed into a fresh EPI300 background. These subclones were streaked on LB plates containing arabinose, SDS (0.2% w/v) and EDTA (3mM) but did not grow under these conditions (Figure 5-6 E & F). This suggests that the resistance is independent of the metagenomic DNA and is most likely due to a chromosomal mutation, which explains why the resistance phenotype is observed regardless of the presence or absence of arabinose. Although the resistance to detergents was interesting in its own right it was unrelated to the metagenomic DNA and as a result these clones were not pursued further.

5.4. Isolating novel exopolysaccharides from the metagenomic DNA library

Because attempts at using positive selection to identify metagenomic clones that were cold-sensitive or produced novel cell envelope components had been unsuccessful, it was decided that a simple screening procedure could prove more effective. Exopolysaccharides (EPS) were good candidates since they were shown to be used as cryoprotectants by some psychrophilic microorganisms (Nichols *et al*, 2005) and because a simple screening method could be devised to screen the metagenomic library for clones that confer exopolysaccharide production in *E. coli*.

5.4.1. Screening the metagenomic library for Calcofluor-bright clones

A simple, high-throughput screen was designed to isolate clones from the metagenomic library that produced exopolysaccharides. Production of EPS can be easily detected by adding Calcofluor White (also called Fluorescence Brightener 28)

to the medium since colonies that produce Calcofluor-binding EPS will fluoresce when exposed to ultraviolet (UV) light. For example, *Sinorhizobium meliloti* produces Calcofluor-binding EPS (Doherty *et al*, 1988) and genetic mutations that result in altered EPS production, such as an *exoS* mutant that overproduces EPS or an *exoY* mutant that does not produce any, can be visualised by screening for altered fluorescence (Figure 5-7 A & B). *E. coli* EPI300 itself does not fluoresce in the presence of Calcofluor (Figure 5-7 C & D), allowing selection for clones that carry a DNA fragment involved in Calcofluor-binding EPS. As seen with *S. meliloti*, different levels of production can give different levels of fluorescence.

In order to screen for library clones that produce Calcofluor-binding EPS, 20,000 clones were spread on LB agar plates containing Calcofluor (0.02% w/v) and arabinose (0.01% w/v), which were then incubated at 37°C overnight. All plates displayed a number of fluorescent colonies when examined under UV light (Figure 5-8 A & B). A total of 80 fluorescent colonies were picked from these plates and patched on LB with Calcofluor and arabinose to confirm the fluorescence phenotype (Figure 5-8 C & D). Of the initial 80 clones isolated, 59 were confirmed as fluorescent and were assigned the names CF01 to CF59. These were retained for further characterisation.

5.4.2. Preliminary characterisation of the Calcofluor-bright metagenomic clones

Because of the way the metagenomic library was constructed, there was a likelihood that some of the 59 Calcofluor-bright clones isolated were duplicates. In order to check for duplicates, the fosmid was extracted from all 59 clones (Section

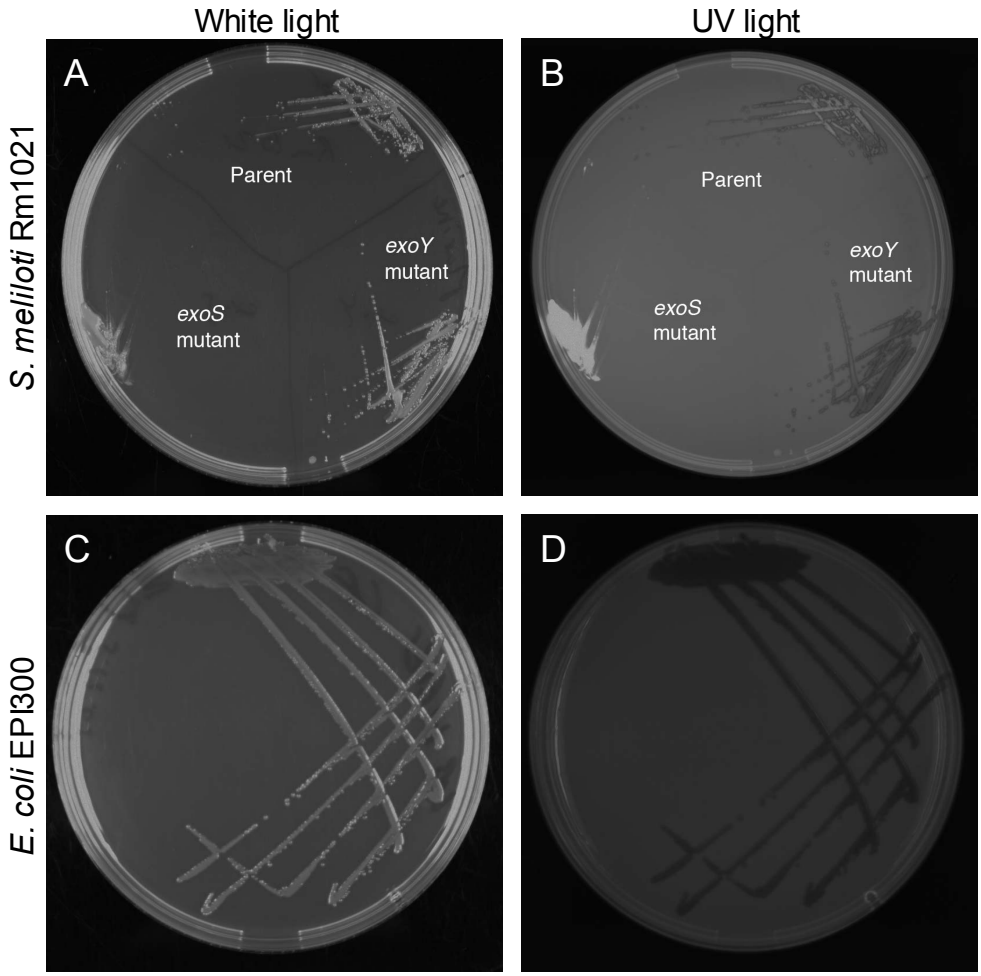


Figure 5-7. Calcofluor fluorescence in the presence of expoloysaccharides. Three *S. meliloti* strains, the parent strain (Rm1021), the *exoS*::Tn*5* mutant (which overproduces EPS), and the *exoY*::Tn*5* mutant (which does not produce any EPS) were streaked on LB agar with Calcofluor (0.02% w/v), incubated at 30°C for 3 days, then visualised under white light (A) and UV (B). *E. coli* EPI300 was streaked on LB agar with Calcofluor (0.02% w/v), incubated at 37°C overnight and then visualised under white light (C) and UV (D).

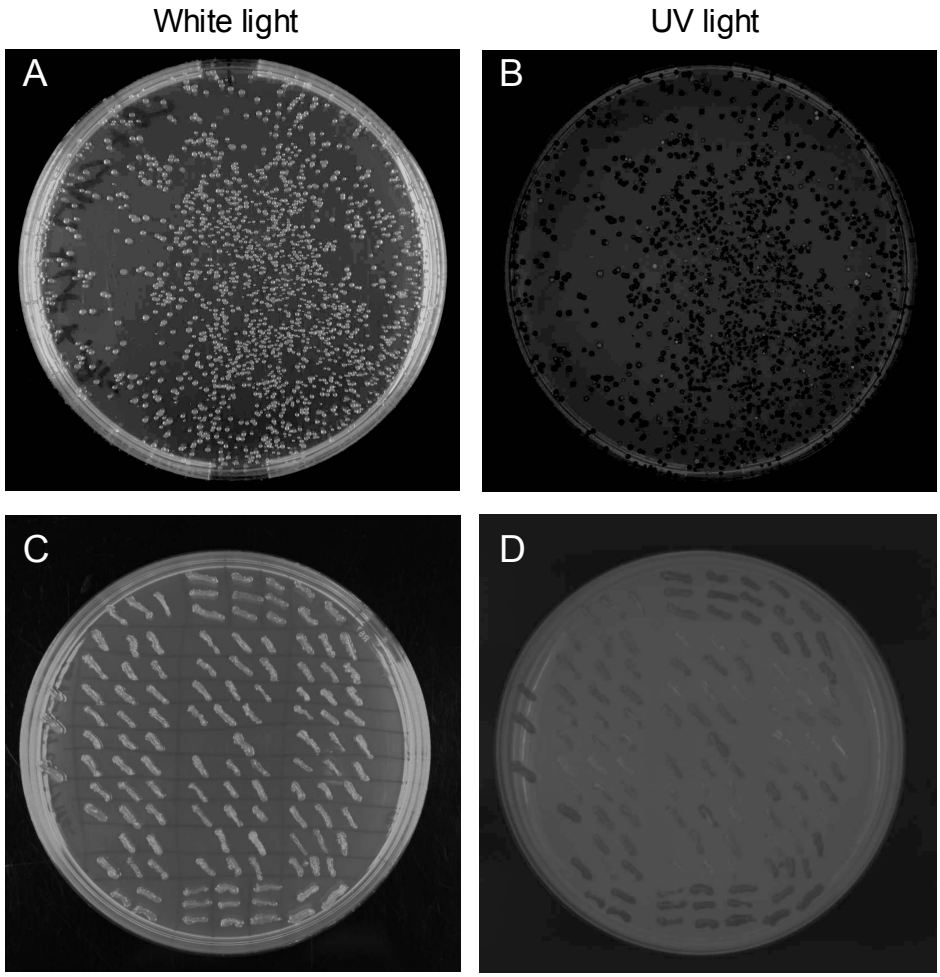


Figure 5-8. Screening for exopolysaccharides from the metagenomic library. Clones from the library were spread on 14 LB agar plates (1490 clones per plate) containing chloramphenicol (12.5μg/ml), Calcofluor (0.02% w/v) and arabinose (0.01% w/v). The plates (one representative is shown) were incubated at 37°C overnight, then visualised under white light (A) and UV (B). 80 fluorescent clones were patched on LB agar supplemented and incubated as above, then visualised under white light (C) and UV (D).

2.3.3), then the fosmid from clones CF01 to CF49 was restricted with EcoRI. The restriction patterns of the fosmids were analysed by agarose gel electrophoresis and compared to identify similar banding patterns. None of the restricted fosmids had identical restriction patterns to each other (Figure 5-9), suggesting that there were no duplicates and that the 49 clones were unique.

To confirm that the Calcofluor fluorescence was conferred by the metagenomic DNA and not random chromosomal mutations, the extracted fosmids from all 59 clones were transformed into a fresh EPI300 background. Each sub-clone was then streaked alongside the original clone on LB containing Calcofluor and arabinose and then visualised under UV light. Each clone and sub-clone was then scored as having low (+), medium (++) or high (+++) fluorescence intensity (Table 5-1) In all cases but one, the clone and subclone both displayed the same level of fluorescence, as seen with four representative example (Figure 5-10 A-D), confirming that the fluorescent phenotype was indeed due to the metagenomic library DNA. The exception, CF33, was interesting because the fluorescence of the subclone was much brighter than that of the original clones (Figure 5-10 E). This was confirmed by repeating the fosmid extraction from the original CF33 clone, transforming into EPI300 and checking the fluorescence of the new subclone. Again the fluorescence of the subclone was much brighter than that of the original clone (Figure 5-10 F). Although the reason for the peculiar phenotype of the FL33 subclones is unclear, in the case of all the other clones the fluorescence was demonstrated to be due to the presence of the fosmids, which provides evidence that the metagenomic DNA in these clones encodes genes involved in production of Calcofluor-binding exopolysaccharides.

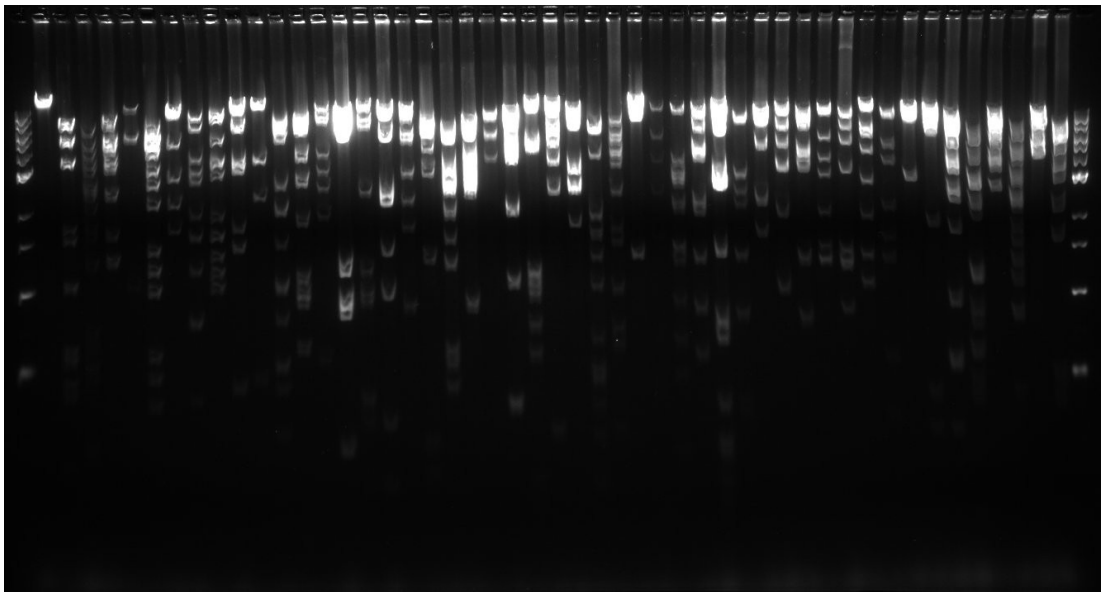


Figure 5-9. Restriction analysis of fosmids from 49 Calcofluor-bright clones. Fosmids were extracted from clones CF01 to CF49, restricted with EcoRI then analysed by agarose gel electrophoresis. The first and last lanes have the 1kb DNA ladder.

Table 5-1. The Calcofluor-fluorescent metagenomic clones

Clone name	Clone	Subclone
CF01	++	++
CF02	++	++
CF03	+++	+++
CF04	+	+
CF05	+++	+++
CF06	+++	+++
CF07	++	++
CF08	++	++
CF09	+++	+++
CF10	+++	+++
CF11	+++	+++
CF12	+	+
CF13	++	++
CF14	+++	+++
CF15	++	++
CF16	+	+
CF17	++	++
CF18	++	++
CF19	+++	+++
CF20	+++	+++
CF21	+++	+++
CF22	++	++
CF23	++	++
CF24	+++	+++
CF25	++	++
CF26	+	+
CF27	+	+
CF28	+	+
CF29	+++	+++
CF30	+	+

Clone name	Original	Transformed
CF31	++	++
CF32	++	++
CF33	+	+++
CF34	+	+
CF35	+	+
CF36	+++	+++
CF37	+++	+++
CF38	+	+
CF39	++	++
CF40	+	+
CF41	+	+
CF42	+++	+++
CF43	+++	+++
CF44	++	++
CF45	+	+
CF46	+++	+++
CF47	+	+
CF48	+++	+++
CF49	+++	+++
CF50	+++	+++
CF51	++	++
CF52	+	+
CF53	+	+
CF54	++	++
CF55	+	+
CF56	+	+
CF57	++	++
CF58	+++	+++
CF59	+	+

+ low fluorescence
++ medium fluorescence
+++ strong fluorescence

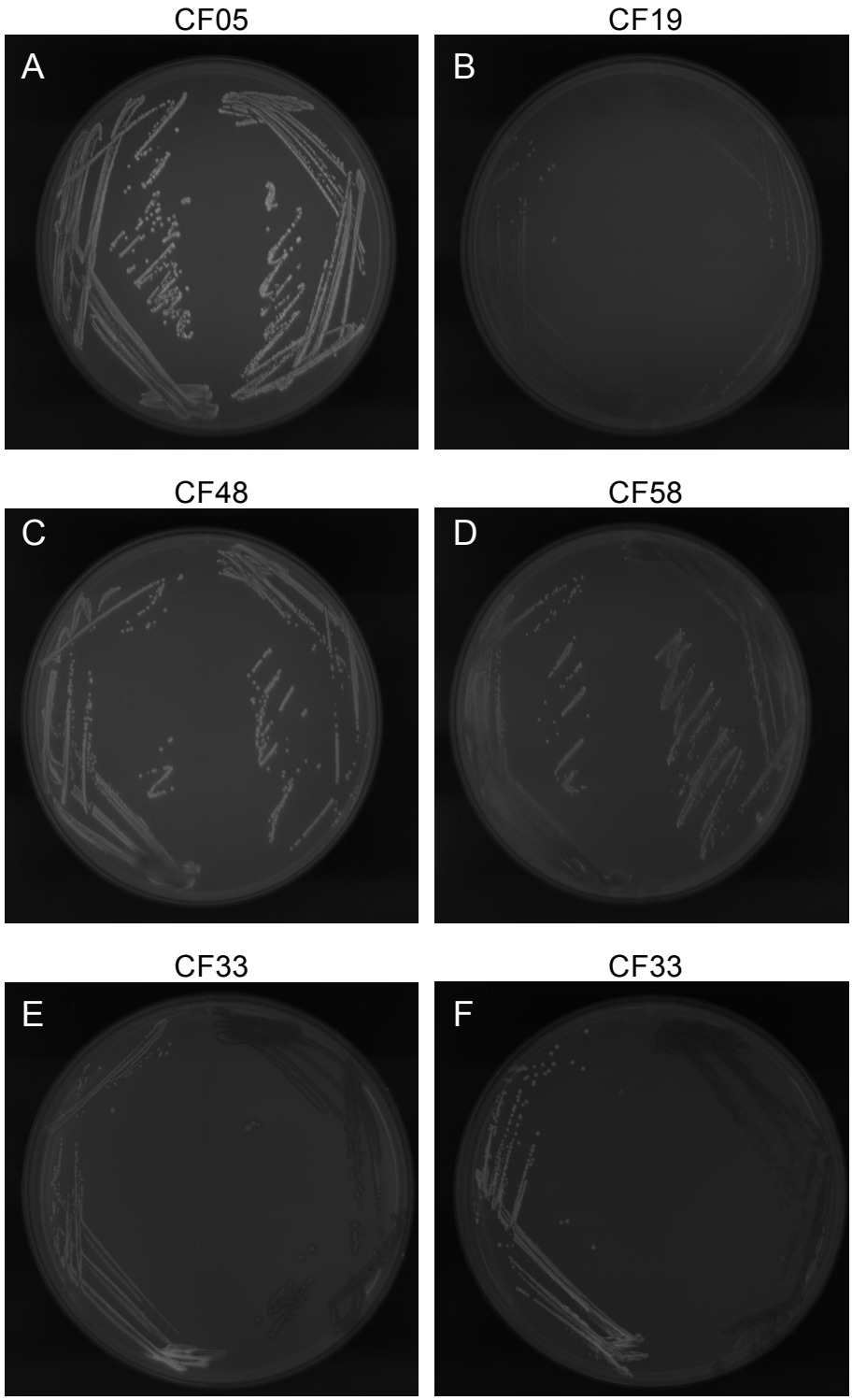


Figure 5-10. Confirming the fluorescence phenotype of the Calcofluor-bright metagenomic clones. After the extracted fosmids had been transformed into a fresh EPI300 background, five subclones (left streak) were streaked, alongside the original clones (right streak), on LB agar plates containing chloramphenicol (12.5µg/ml), Calcofluor (0.02% w/v) and arabinose (0.01% w/v). The plates were incubated at 37°C overnight then visualised under UV. Two separate attempts made with the CF33 clone are shown.

5.5. Using metagenomic techniques to identify cell envelope mutants in *P. profundum* SS9

Although neither of the *P. profundum* SS9 proteins investigated in this study are involved in LPS synthesis, other mutants isolated from the *P. profundum* SS9 mutant library (Section 1.2.1) were shown to have robust cold-sensitive phenotypes and had clear LPS alterations when analysed by SDS-PAGE (Allcock DJ and Ferguson GP, unpublished data). This suggests that intact membrane polysaccharides are required for cold temperature growth of *P. profundum* SS9 and confirms LPS analysis of cold-sensitive mutants as a valid method to investigate the role of these polysaccharides in cold-temperature adaptation of *P. profundum* SS9. One approach to investigate this adaptation could be to isolate new *P. profundum* SS9 mutants with alterations in their cell envelope by applying the selection and screening methods used earlier in this chapter.

5.5.1. Identifying genes involved in EPS biosynthesis in P. profundum SS9

P. profundum SS9 was originally isolated from an amphipod homogenate, which raises the possibility that it forms an interaction with the amphipod (DeLong, 1986). Biofilm formation has been shown to be an important feature of symbiotic as well as pathogenic life styles (Ramey *et al*, 2004) and biofilm formation is usually associated with the production of EPS (Van Houdt and Michiels, 2005).To determine if SS9R can produce Calcofluor-binding EPS, the strain was grown on Marine agar at 0.1MPa and 15°C in the presence of Calcofluor. After growth under these conditions, SS9R was fluorescent when visualised under UV, which shows that *P.*

profundum SS9 can produce Calcofluor-binding EPS on Marine agar (Figure 5-11). By using random mini-Tn*5* transposon mutagenesis, and screening for mutants with altered fluorescence under UV when grown on Marine agar with Calcofluor, it would be possible to identify genes involved in the biosynthesis of EPS. This approach could allow the reconstruction of the entire EPS pathway in *P. profundum* SS9, as was successfully done to characterise the EPS pathway in *S. meliloti* (Reuber *et al*, 1991).

5.5.2. Identifying genes involved in resistance to detergents in P. profundum SS9

As mentioned previously, outer membrane polysaccharides confer increased resistance to detergents in Gram-negative bacteria (Nixdorff *et al*, 1977). Additionally, the composition of the cell envelope of *P. profundum* SS9 is regulated in response to high pressure and cold temperature (Bidle and Bartlett, 2001). Investigating genes involved in cell envelope integrity (including resistance to detergents) could shed light on the ability of *P. profundum* SS9 to grow under these extreme conditions. The resistance of SS9R to the detergents DOC and SDS was therefore investigated. SS9R was found to be resistant to 0.2% SDS when grown on Marine agar at 0.1MPa and 15°C (Figure 5-12 A & B). Since EDTA had been shown to increase sensitivity to SDS (Figure 5-5), it was again used here in conjunction with SDS. Although EDTA by itself inhibited the growth of SS9R, combining both compounds had a greater inhibitory effect on the growth of SS9R (Figure 5-12 B & C, respectively). Increasing the concentration of either compound prevented the growth of SS9R on Marine agar (Figure 5-12 E & F), so 0.2% SDS with 3mM

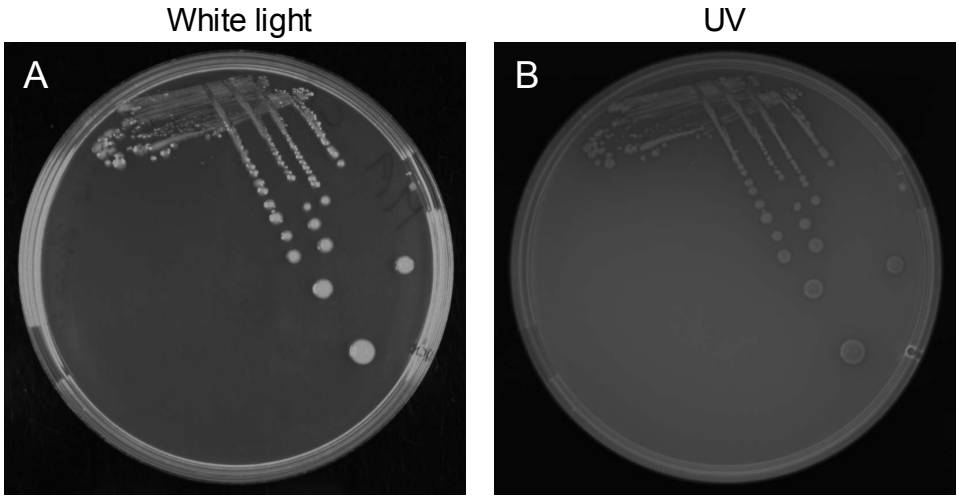


Figure 5-11. Production of Calcofluor-binding EPS by *P. profundum* SS9. Strain SS9R was streaked on Marine agar supplemented with HEPES (100mM), glucose (20mM) and Calcofluor (0.02% w/v). The plate was incubated at 0.1MPa and 15°C for 96 hours, then visualised under white light (A) and UV (B).

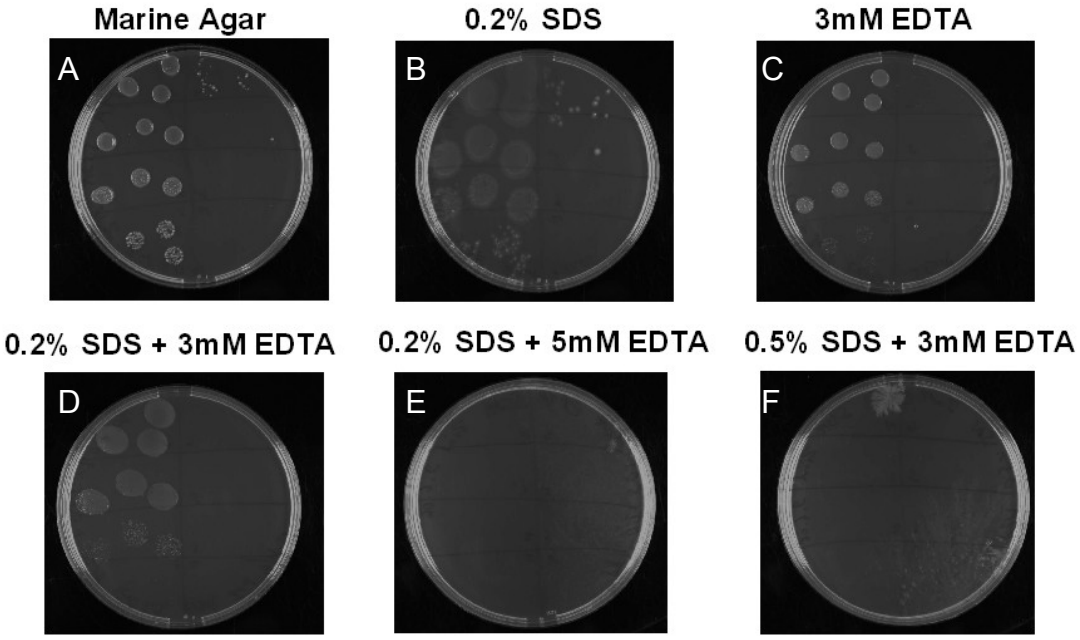


Figure 5-12. Resistance of *P. profundum* SS9R to SDS. A liquid culture of SS9R was grown for 40 hours, diluted to an OD_{600} of 0.2, serially diluted (10^{-1}-10^{-6}) and spotted in triplicate on Marine agar with HEPES (100mM) and glucose (20mM), in the presence of SDS (0.2% w/v), EDTA (2mM), or both, as indicated. Plates were incubated at 0.1MPa and 15°C for 96 hours.

EDTA was chosen as the borderline concentration. Random mini-Tn*5* mutagenesis can be used to identify genes involved in resistance to detergents, by screening for mutants that no longer grow on Marine agar supplemented with 0.2% SDS and 3mM EDTA.

On Marine agar, which is used to grow *P. profundum* SS9 routinely in the lab, this screen could only be carried out with SDS as a detergent because DOC precipitated when added to Marine agar (Figure 5-13). Since DOC does not precipitate in LB, an LB derivative was developed that could support the growth *P. profundum* SS9. Comparing the composition of Marine Broth and LB revealed that Marine Broth had a higher content of sodium chloride, calcium and magnesium. LB/MC, used to grow *S. meliloti* routinely, is LB supplemented with 2.5mM $MgSO_4$ and 2.5mM $CaCl_2$. By supplementing LB/MC with 0.17M NaCl, 100mM HEPES and 20mM glucose, it was possible to grow SS9R on this modified medium, LB/MCSG (Figure 5-14). SS9R was grown on LB/MCSG with 20mM DOC, and this was enough to significantly inhibit growth (Figure 5-15). Since DOC by itself had such an inhibitory effect, EDTA was deemed unnecessary and was not used. In summary, borderline concentrations for the detergents were 20mM DOC, or 0.2% SDS and 3mM EDTA, and these could be used to screen for *P. profundum* SS9 mutants with increased sensitivity to detergents.

5.6. Discussion

The metagenomic DNA library provided an opportunity to identify novel polysaccharides from marine microorganisms. Aside from potential biotechnological or pharmaceutical interest, novel materials could be involved in adaptation of certain

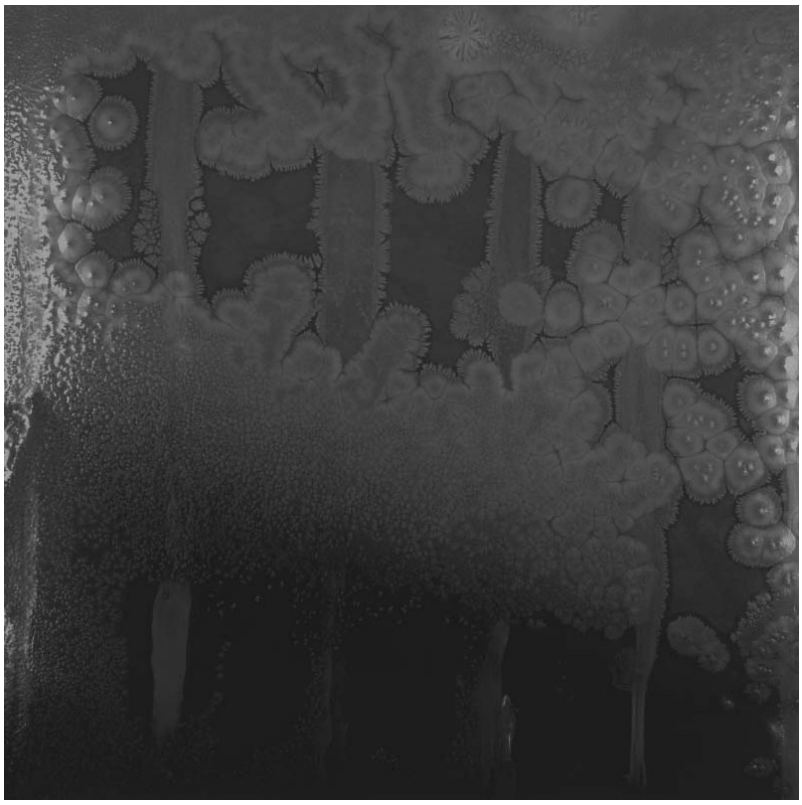


Figure 5-13. DOC precipitation in Marine agar. Marine agar was supplemented with 20mM DOC before pouring. DOC immediately precipitates upon addition to the agar and forms crystals after the agar has set.

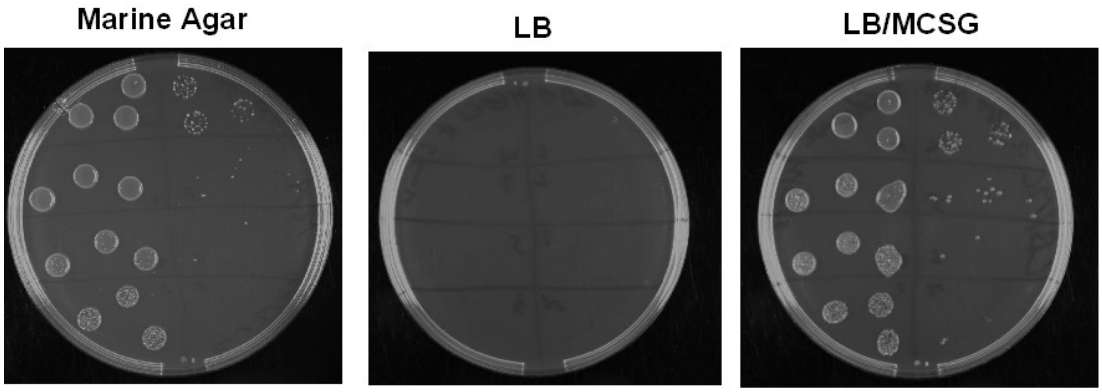


Figure 5-14. *P. profundum* SS9R grown on LB agar. A liquid culture of SS9R was grown for 40 hours, diluted to an OD_{600} of 0.2, serially diluted (10^{-1}-10^{-6}), then the dilutions were spotted in triplicate on Marine Agar (supplemented with 100mM HEPES and 20mM glucose), LB agar, and LB/MCSG agar (LB agar supplemented with 2.5mM $MgSO_4$, 2.5mM $CaCl_2$, 0.17M NaCl, 100mM HEPES, 20mM glucose). Plates were incubated at 15°C for 96 hours.

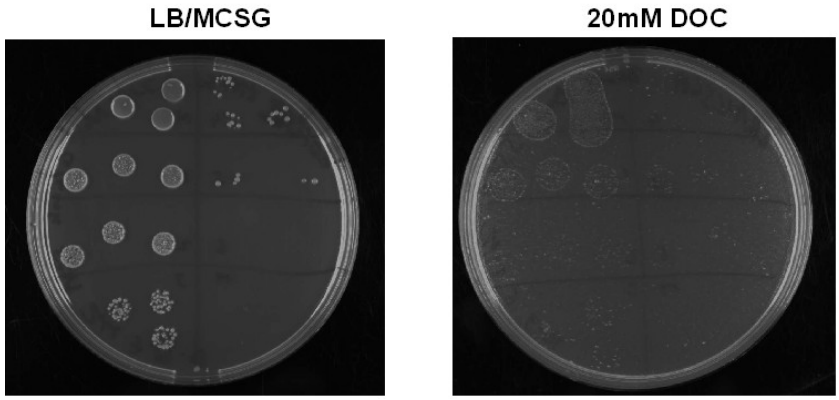


Figure 5-15. Sensitivity of *P. profundum* SS9R to DOC. A liquid culture of SS9R was grown for 40 hours, diluted to an OD_{600} of 0.2, serially diluted (10^{-1}-10^{-6}) and spotted in triplicate on LB agar with 0.33M NaCl $MgSO_4$ (2.5mM), $CaCl_2$ (2.5mM), HEPES (100mM) and glucose (20mM), in the presence or absence of DOC (20mM). Plates were incubated at 0.1MPa and 15°C for 96 hours.

microorganisms to specific conditions, and studying these products could further our understanding of extremophiles and their evolutionary adaptations. Additionally, the techniques used with the library could also be applied to isolate cell envelope mutants in *P. profundum* SS9 and gain further understanding of the deep-sea adaptation of this organism.

The 16S rRNA sequences from the library were aligned and used to construct a phylogenetic tree. This tree illustrated the wide diversity of microorganisms represented in the library, since these organisms spanned most of the major bacterial groups. More importantly, many metagenomic clones are closely related to uncultured organisms, and are probably uncultured themselves. This highlights the importance of metagenomics in isolating novel products from uncultured organisms.

5.6.1. Novel polysaccharides from a marine metagenomic library

A metagenomic clone encoding a cell envelope modification could be isolated by selecting for clones with increased resistance to detergents. This would allow selection for novel polysaccharides that alter the envelope. Although 3 clones with increased resistance were selected, further investigation showed the phenotype was not linked to metagenomic DNA and was probably the result of a random chromosomal mutation. Another approach, using Calcofluor to screen for EPS production, yielded 59 metagenomic clones that conferred fluorescence on the *E. coli* host. The phenotype of 58 of these clones was confirmed as being linked to the metagenomic DNA. The genes present on these clones are thought to be either responsible for production of EPS or for activating cryptic EPS production pathways in *E. coli* EPI300.

The CF33 clone displayed an intriguing behaviour, since subcloning the fosmid consistently resulted in increased fluorescence compared to the original. One possibility is that a suppressor mutation present in the original clone reduces Calcofluor-binding exopolysaccharide production. When the fosmid is moved into a fresh background lacking this chromosomal mutation, the fluorescence phenotype is then expressed fully.

Having confirmed that the production of Calcofluor-binding EPS is conferred by the metagenomic DNA, the EPS themselves will be further investigated, either by determining their composition using mass spectrometry, or by studying their flexibility and material deformation stability using a rheometer. Industrially significant characteristics can be investigated, such as elasticity, adhesiveness. Once interesting properties have been found, the DNA fragments could be further analysed to determine which gene or set of genes are involved in EPS production, and whether the pathway is chimeric and involves *E. coli* molecules provided by the EPI300 host.

Although the clones isolated as cold-resistant did not display a robust phenotype and were subsequently discarded, the techniques used in this study could still be applied to the isolation of cell envelope components and ultimately applied to deep-sea samples. Constructing a metagenomic library from a deep-sea water sample and screening or selecting for clones which alter the cell envelope polysaccharides of the host may give us valuable insights into deep-sea adaptation. One exciting possibility is that some of these clones may code for a minimal set of genes that could increase the resistance of piezosensitive organisms, such as *E. coli*, to high pressure, in the same way that GroEL from a psychrophile conferred cold adapted growth to *E. coli* (Ferrer *et al*, 2003).

5.6.2. Identifying cell envelope components in P. profundum SS9

P. profundum SS9 produces Calcofluor-binding EPS when grown on Marine agar. Random transposon mutagenesis could therefore be used to identify the components involved in EPS synthesis by screening for *P. profundum* SS9 mutants with altered Calcofluor fluorescence. This approach has been successfully used to characterise the synthesis pathway of EPS in *S. meliloti* (Reuber *et al*, 1991). This may further our understanding of the role of EPS in cold adaptation.

In order to screen for mutants with increased sensitivity to detergents, borderline conditions at which SS9R can grown in the presence of these detergents had to be determined. This was done for a combination of SDS and EDTA on Marine agar. DOC precipitates in Marine agar, but a modified LB agar that closely mimics the composition of Marine agar supported the growth of SS9R at levels comparable to Marine agar. A borderline concentration of DOC was found at which SS9R can still grow on LB/MCSG.

With the borderline concentrations established, random transposon mutagenesis can be used to screen for *P. profundum* SS9 mutants with increased sensitivity to these detergents. These mutants could then be further analysed for alterations in their LPS or other cell surface polysaccharides. Ultimately this selection process can be adapted and used in conjunction with the low gelling temperature agar overlay method to screen for sensitivity to detergents at high pressure. If any of these mutants display a cold temperature or high pressure sensitivity then a link can be established between these extremes and specific cell envelope components, which will further our understanding of microbial deep-sea adaptation.

Chapter 6

Concluding remarks

P. profundum SS9 is a piezophilic and psychrophilic bacterium that is used as a model organism to study bacterial deep-sea adaptation. *P. profundum* SS9 grows optimally at 28MPa and 15°C but can grow over a range of temperatures (2-20°C) and pressures (0.1-90MPa) (DeLong, 1986). Two *P. profundum* SS9 mini-transposon mutants, FL23 and FL28, had previously been characterised as pressure-sensitive and pressure-enhanced, respectively, at 45MPa and 17°C (Lauro *et al*, 2008). FL23 carries a mini-Tn*10* insertion in the *pbpra3229* gene, whose product is annotated as a phosphoheptose isomerase (GmhA) but shares higher sequence homology to *E. coli* DiaA, a DnaA initiator-associating factor required for timely initiation of DNA replication (Ishida *et* al, 2004). FL28 carries a mini-Tn*10* insertion in the *pbpra1039* gene, whose product is annotated as SeqA, a negative regular of DNA replication that is also essential for timely initiation of replication (Lu *et al*, 1994). This raised the possibility that regulation of initiation of DNA replication is critical for pressure adaptation in *P. profundum* SS9 and the primary goal of this project was to test this hypothesis.

This study has shown that Pbpra3229 is not a GmhA, but is instead a PpDiaA, whereas Pbpra1039 was confirmed as being a PpSeqA. The absence of PpDiaA leads to loss of high pressure adaptation in FL23 on solid agar at 28MPa and 15°C, whereas loss of PpSeqA leads to pressure-enhanced growth of FL28 at 0.1MPa compared to 28MPa. This provides evidence that timely initiation of DNA replication is critical for the ability of *P. profundum* SS9 to adapt to different

pressures. More specifically, a positive regulator of DNA replication, such as DiaA, is required for adaptation to high pressure whereas a negative regulator of replication, such as SeqA, is essential for adaptation to atmospheric pressure. Although loss of PpDiaA causes high pressure sensitivity in FL23, PpDiaA itself does not seem to be a pressure-adapted protein, since EcDiaA rescued the mutant phenotype of FL23 at 28 and 45MPa to the same extent as PpDiaA. Interestingly, PpSeqA was able to complement the filamentation phenotype of the *E. coli seqA* mutant at 15°C but not 37°C, which suggests that PpSeqA could be a cold-adapted protein.

Although FL23 and FL28 showed decreased viability compared to SS9R under all conditions observed, the *E. coli diaA* and *seqA* mutants have no change in viability despite the disruption in timely initiation of DNA replication. One likely explanation is that the loss of timely initiation in one chromosome in *P. profundum* SS9 disrupts the synchrony between the two chromosomes and leads to decreased viability, whereas *E. coli* has only one chromosome so loss of timely initiation does not affect viability. This theory seems to be supported by a study in *V. cholerae* were overproduction of SeqA disrupted synchrony and lead to decreased viability (Saint-Dic *et al*, 2008). Another piece of evidence comes from RctB, the DNA replication initiator protein of Chromosome II. *P. profundum* FL31, which encodes a partial RctB protein, grows similarly to the parent at 0.1MPa and 15°C but filaments and forms abnormal cellular morphologies at 45MPa and 15°C. Comparing FL23, FL28 and FL31 shows that disrupting initiation of DNA replication of either chromosome decreases cell viability in *P. profundum* SS9.

The following is the proposed model for timely initiation of DNA replication

and the effect of asynchronous replication on pressure adaptation in *P. profundum* SS9 (Figure 6-1). Loss of the replication promoter PpDiaA disrupts timely initiation of Chromosome I replication, the resulting asynchronous replication of the chromosomes is exacerbated at high pressure. Loss of the negative regular PpSeqA also disrupts timely initiation of Chromosome I replication, but in this instance the asynchrony is exacerbated at atmospheric pressure. RctB is an essential protein but a truncated protein lacking the C-terminus is functional at atmospheric pressure, although it is unable to properly initiate replication of Chromosome II at high pressure and this leads to asynchrony. Therefore it is hypothesised that disrupting a replication promoter of either chromosome results in asynchrony at high pressure, whereas disruption of a negative regulator results in asynchrony at atmospheric pressure and a pressure-enhanced phenotype. Additional evidence to support this model could be generated by directly examining initiation of DNA replication in *P. profundum* SS9, either by adapting flow cytometry to work in this organism or by using operator sequence arrays in the origin of replication. Isolating purified DiaA, SeqA and RctB proteins from *P. profundum* SS9 would allow detailed biochemical analysis of their activity and interaction with other replication proteins. Further work on initiation of replication of the two chromosomes in *P. profundum* SS9 will expand the model and allow a more detailed understanding of initiation of DNA replication in *P. profundum* SS9 and its role in deep-sea adaptation.

This project was funded in part by the Leverhulme Trust, who support research into the development of novel materials. Clones producing Calcofluor-binding exopolysaccharides were isolated from a marine metagenomic library and are awaiting further compositional and rheological characterisation. If some of them

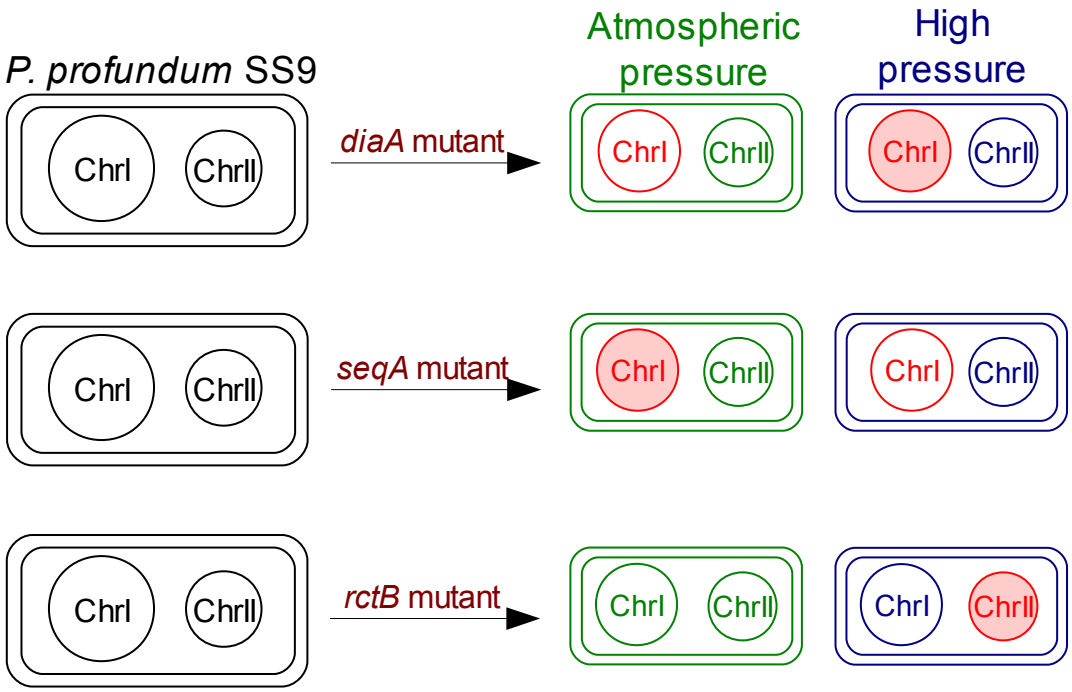


Figure 6-1. Timing of DNA replication and its effect on the high pressure growth of *P. profundum* SS9. Green represents cells at atmospheric pressure, blue at high pressure. The *P. profundum* SS9 chromosome with mistimed replication is drawn in red, shading represents exacerbated defect.

have unique properties they could help in advancing the Leverhulme Trust's goal to identify novel polysaccharides. Additionally the techniques developed can be used to construct and screen a deep-sea metagenomic library as well as to identify polysaccharides involved in deep-sea adaptation in *P. profundum* SS9. Either approach would further our knowledge of microbial adaptation to the deep sea.

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Appendix

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Insights into the molecular basis of piezophily from genetic studies on the deep-sea bacterium, Photobacterium profundum SS9

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Insights into the molecular basis of piezophily from genetic studies on the deep-sea bacterium, *Photobacterium profundum* SS9

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Abstract

The deep-sea bacterium, *Photobacterium profundum* SS9 has been adopted as model organism to understand the molecular basis of cold-adapted high pressure-loving (piezophilic) growth. Despite growing optimally at 28 MPa (15°C), *P. profundum* SS9 can grow over a wide range of pressures and temperatures. The ability to grow at atmospheric pressure has enabled a limited set of genetic tools to be developed, which has provided genetic insights into the mechanism of piezophilic growth in *P. profundum* SS9. This review focuses on how genetic studies have uncovered the importance of processes affecting the DNA and the bacterial cell envelope in the piezophilic growth of *P. profundum* SS9. In addition, a method was developed to assess quantitative piezophilic colony growth of *P. profundum* SS9 on solid agar. Future studies, using this methodology, could provide novel insights into the molecular basis of piezophilic, surface-attached growth.

Key words; Cell envelope, DNA replication, RecD, H-NS, Piezophily,

Introduction

In contrast with research into other types of extremophiles, studies are lacking on pressure-loving microorganisms known as piezophiles [1]. However, since ~70% of the earth's surface is covered with oceans, which have an average temperature of 3°C and exert an average pressure of ~38 MPa (atmospheric pressure=0.1 MPa), the ability to grow optimally at high pressure is crucial for many marine microorganisms, particularly deep-sea microorganisms. To gain a better understanding as to the mechanisms governing cold-adapted piezophilic growth, the deep-sea bacterium, *Photobacterium profundum* SS9 has been adopted as a model organism [2]. *P. profundum* SS9 is a γ-proteobacterium, which was originally isolated from an amphipod homogenate, obtained from the Sulu sea in the Philippines from a depth of 2.5 km (9°C) [3]. Although *P. profundum* SS9 grows optimally at 28 MPa (15°C), it can grow over a wide range of pressures (0.1-90 MPa) and temperatures (2-20°C). The ability to grow at atmospheric pressure has enabled the development of a limited set of genetic tools for *P. profundum* SS9 [4-10]. The *P. profundum* SS9 genome has also been sequenced and annotated and consists of two chromosomes, chromosome I and II, which are 4.1 and 2.2 Mbp, respectively [11]. *P. profundum* SS9 also contains an 80 kb plasmid, which is dispensable for piezophilic growth. The *P. profundum* SS9 genome encodes 15 rRNA operons, which is the highest number known for bacterial species. The

high number of rRNA operons is thought to enable *P. profundum* SS9 to rapidly adapt to changing environmental conditions.

Microarray studies revealed that *P. profundum* SS9 undergoes major changes in gene expression when the pressure is shifted between 0.1 and 28 MPa [11, 12]. In contrast to the piezo-tolerant bacterium *E. coli*, which has increased levels of chaperones when grown at high pressure [13], the transcription of chaperone genes is up regulated in *P. profundum* SS9 growing at atmospheric pressure [11]. Additionally, the transcription of DNA repair genes is also up regulated in *P. profundum* SS9 at atmospheric pressure. Combined these findings, show that atmospheric pressure, rather than high pressure, induces stress response genes in *P. profundum* SS9 adding further evidence that this bacterium is truly piezophilic.

Genomic comparisons of *P. profundum* SS9 with two other *P. profundum* strains, the piezo-sensitive strain (3TCK) and the piezophilic strain (DSJ4) revealed that 171 genes appear to be absent in the piezo-sensitive strain but present in both piezophilic strains [12]. This suggests that at least some of these gene products could be important in piezophilic growth. However, future genetic studies will be necessary to confirm the importance of these gene products in the piezophilic growth of *P. profundum* SS9 and/or DSJ4. In this review, we will focus on insights that have been gained into the mechanism of piezophilic growth from genetic studies in the cold-adapted piezophile, *P. profundum* SS9. In particular we will focus on the importance of processes affecting the DNA and the bacterial cell envelope. Finally, we will outline a method for the quantitative analysis of *P. profundum* SS9 colony growth at high pressure.

2. Genetic insights into the mechanism of piezophilic growth

2.1 Importance of processes affecting the DNA

The importance of DNA repair in the piezophilic growth of *P. profundum* SS9 came from the characterization of the first piezo-sensitive chemically-induced mutant, which had a point mutation in the *recD* gene resulting in an internal stop codon [6, 7]. The subsequent construction and characterization of two additional *P. profundum* SS9 *recD* insertional mutants then confirmed the importance of RecD in piezophilic growth [6]. In *E. coli*, RecD forms the RecBCD enzyme and is the major pathway for the repair of DNA double-strand breaks and homologous recombination [14]. RecBCD unwinds the DNA at the double-strand break and RecD is a rapid helicase, which moves on the 5' end of the DNA strand [15]. Therefore, the finding that RecD is important in the piezophilic growth of *P. profundum* SS9 [6, 7] suggests that high pressure induces DNA damage, which is normally repaired by RecD. In the absence of RecD, the DNA damage is not repaired and *P. profundum* SS9 becomes piezo-sensitive.

At high pressures (up to 50 MPa), *E. coli* cells remain viable but form filaments and loose their ability to maintain plasmids [6]. Interesting, the *P. profundum* SS9 *recD* but not the *E. coli recD* could prevent high pressure-induced filamentation of cells and the loss of plasmids in *E. coli* lacking RecD. Since the *P. profundum* SS9 RecD protein, but not the *E. coli* RecD protein, was able to impart novel functions on *E. coli* lacking RecD at high pressure, this suggests the *P. profundum* SS9 RecD protein may possess specific high pressure-adaptations that are lacking in the *E. coli* RecD protein. It was suggested that a 64 amino acid structural motif present in the *P. profundum* SS9 RecD protein but not the *E. coli* RecD protein could be involved in the high pressure functions.

Further evidence that processes involving the DNA appear to be important in the piezophilic growth of *P. profundum* SS9 has also been obtained recently from a transposon screen for high pressure-sensitive mutants [10]. Mini-Tn*10* and mini-Tn*5* transposon mutant libraries were constructed in the rifampicin-resistant mutant of *P. profundum* SS9, known as SS9R and were then screened for piezo-sensitivity by comparing their growth at 45 versus 0.1 MPa (17°C). By using this approach, a number of *P. profundum* SS9R transposon insertion high pressure-sensitive mutants were identified. Two of the genes disrupted in these transposon mutants are predicted to encode homologs of *E. coli* DiaA and *Vibrio cholerae* RctB, which are involved in the initiation of DNA replication [16, 17]. In *E. coli*, ATP bound DnaA binds to the DnaA box at the origin of replication (*oriC*) and DiaA (DnaA Initiator Associating Factor) binds to multiple molecules of DnaA and promotes this process resulting in the synchronous initiation of DNA replication [17, 18]. In contrast, in *E. coli* lacking *diaA*, the initiation of DNA replication is asynchronous resulting in odd numbers of chromosomes per cell [17]. The *E. coli* DiaA protein can therefore be viewed as a positive regulator of the initiation of DNA replication [18]. Despite the asynchronous DNA initiation phenotype of an *E. coli diaA* mutant, there appears to be no impact on the growth [17]. Consequently, if the gene disrupted in the piezo-sensitive *P. profundum* SS9R transposon mutant encodes a DiaA homolog [10], this supports a model whereby the initiation of DNA replication in *P. profundum* SS9 is pressure sensitive and that the promotion of this process by DiaA is important for piezophilic growth (Fig. 1). However, as mentioned earlier, unlike *E. coli*, which has one chromosome, *P. profundum* SS9 has two different chromosomes [11]. In the closely related bacterium, *V. cholera*, which also has two different chromosomes, replication at the origin of chromosome I is DnaA-dependent whereas replication at the origin of chromosome II is independently controlled [19]. Since the genome sequence predicts that the origins of replication of *P. profundum* SS9 chromosomes are similar to those of *V. cholera* [11], this suggests that DiaA is likely to only affect the replication of chromosome I in *P. profundum* SS9.

In contrast to DiaA, the RctB protein is specifically required for the initiation of chromosome II replication in *V. cholerae* [19]. Consistent with its essential function, *rctB* is an essential gene in *V. cholera* [16]. Since the transposon insertion in the *P. profundum* SS9R piezo-sensitive mutant is in the 3' end of the potential *rctB* open reading frame [10], this suggests that it encodes a partially active protein. Although further experiments will be necessary to determine whether the disrupted gene encodes an RctB homolog, these data add support for a model whereby the initiation of chromosome II replication is also high pressure sensitive and that a fully active RctB protein is essential to ensure the correct initiation of chromosome II in *P. profundum* SS9 under these conditions.

Further evidence in support of a model for the importance of the initiation of chromosome I in the piezophilic growth of *P. profundum* SS9 was also obtained from a screen for mutants with high pressure-enhanced growth at 45 versus 0.1 MPa (17°C) [10]. One of the mutants obtained from this screen contains a transposon insertion in a gene encoding a putative SeqA homolog. In *E. coli*, SeqA binds to hemimethylated *oriC* and prevents the binding of DnaA to the origin [20]. Therefore, in contrast to DiaA, which is a positive regulator, SeqA is a negative regulator of the initiation of DNA replication in *E. coli*. If the gene disrupted in the *P. profundum* SS9 transposon mutant encodes a SeqA homolog [10], this would add further support for a model whereby the initiation of DNA replication is sensitive to high pressure and either the presence of a positive regulator of this process or the absence of a negative regulator is important for piezophilic growth (Fig. 1).

A putative H-NS transposon insertion mutant was also isolated as having high pressure-enhanced growth [10]. In *E. coli*, H-NS is a histone-like nucleoid structuring protein, which acts to bridge the DNA duplex and is involved in condensing the bacterial nucleoid [21]. Additionally, H-NS is a global regulator of gene expression and regulates the expression of ~5% of the genes in the *E. coli* genome [22]. Therefore, if the gene disrupted in the *P. profundum* SS9 transposon mutants encodes an H-NS homolog [10], the less-ordered structure of the DNA in the absence of H-NS maybe more favourable at high pressure and/or changes in gene expression in absence of H-NS may also lead to a high pressure-enhanced growth phenotype.

2.2 Importance of processes affecting the cell envelope

One of the first indications of the importance of the cell envelope in the piezophilic growth came from the finding that insertions in an *rpo*E-like locus result in piezo-sensitivity in *P. profundum* SS9 [8]. In *E. coli*, the *rpoE* gene encodes the alternative sigma factor σ^E, which controls the initiation of genes involved in the extracytoplasmic stress response [23]. σ^E is constitutively expressed but maintained in an inactive state in the cytoplasmic membrane through a complex with RseB and the anti-sigma factor, RseA. Under conditions, which result in the accumulation of denatured outer membrane proteins in the periplasm, σ^E is activated by the cleavage of RseA. The genes under control of σ^E encode proteins involved in the re-folding and degradation of outer membrane proteins and the biosynthesis of a number of cell envelope components. Since the accumulation of misfolded proteins in the periplasm is toxic for bacterial growth, the re-folding of these proteins or their degradation is important for growth. Therefore, this suggests that high pressure may result in an increase in the misfolding of outer membrane proteins and that activation of an RpoE-like locus is important to maintain the integrity of the *P. profundum* SS9 cell envelope under these conditions.

A putative *rseB P. profundum* SS9R transposon mutant was also isolated from the high-pressure sensitivity screen [10]. Since in *E. coli*, RseB helps RseA to sequester σ^E in the membrane in the inactive state [23], this suggests that the constitutive activation of σ^E is detrimental for growth of *P. profundum* SS9 at high pressure. However, it has also been suggested that in *E. coli* RseB detects mislocalized lipoproteins in the cell envelope and then induces the σ^E response [24]. Therefore, the specific activation of σ^E by mislocalised lipoproteins during growth at high pressure maybe important for optimum growth.

In addition to changes in membrane proteins, *P. profundum* SS9 also adapts its membrane phospholipids fatty acids in response to pressure changes [5]. Although both polyunsaturated and monounsaturated fatty acids increase as the growth pressure increases, only the latter play an important role in piezophilic growth. For example, in the absence of FabF, *P. profundum* SS9 is unable to up regulate cis-vaccenic acid (C18:1) in response to pressure changes and is defective in piezophilic growth [4]. It is proposed that monounsaturated fatty acids help to maintain the fluidity of the *P. profundum* SS9 membrane at high pressure. In contrast, a *P. profundum* SS9 mutant, which is defective in polyunsaturated fatty acid biosynthesis, is unaffected in piezophilic growth [25].

To date, little is known about the role of other cell envelope components such as lipopolysaccharide and capsular polysaccharide in the piezophilic growth of *P. profundum* SS9. Lipopolysaccharide forms the outermost leaflet of the outer membrane and consists of O-antigen, a core and lipid A [26]. In contrast, capsular polysaccharide CPS is thought to be

composed solely of sugar residues and is tightly associated with the outer surface of the outer membrane [27]. A number of *P. profundum* SS9R with transposon insertions in putative lipopolysaccharide and capsular polysaccharide biosynthesis genes were obtained from a screen for cold sensitive mutants, which were defective at growth at 4°C (0.1 MPa) relative to 15°C (0.1 MPa) [10]. Unlike the screen for high pressure-sensitive mutants, which was performed in liquid culture, the screen for cold-sensitive mutants was performed on solid agar. Interestingly, a number of the cold-sensitive transposon mutants were subsequently found to only show a cold-sensitivity phenotype on solid agar and not in liquid culture. These findings suggests that the mechanisms that *P. profundum* SS9 uses for growth at cold temperatures is dependent upon the mode of growth (i.e. whether it is growing in liquid culture or growing attached to a surface such as solid agar)

2.3 High pressure colony growth

Since the mechanisms used by *P. profundum* SS9 for growth at cold temperatures are dependent upon the mode of growth [10], this also raises the possibility that the mechanisms required for the piezophilic growth of *P. profundum* SS9 may also be affected by the mode of growth. To date, all the piezo-sensitive and piezo-enhanced *P. profundum* SS9 mutants studied have been analysed in terms of their high pressure-growth in liquid culture [4-6, 8, 10]. However, further insights may be gained into piezophily by characterizing the growth of *P. profundum* SS9 mutants on solid agar. Additionally, *P. profundum* SS9 is a member of the *Vibrionaceae* and many *Vibrio* species are known for form host interactions [28]. Since *P. profundum* SS9 was originally isolated from an amphipod homogenate from the deep-sea [3], it is possible that it forms some form of host interaction and the mechanisms involved in piezophilic surface-attached growth maybe more relevant to its life within the deep-sea.

To investigate the growth of *P. profundum* SS9 on solid agar at high pressure, we initially tried a previously developed method, which had been successfully used to analyze the growth of the piezophilic bacterium, *Shewanella* sp strain DSS12 on solid agar at high pressure [29]. In brief, this method involved replica-plating colonies of strain DSS12 on 2.5% (w/v) marine agar and then overlaying with the 2.5% (w/v) low temperature-gelling agar. The agar sandwich was removed from the Petri dish, heat-sealed in a sterilised plastic pouch and after incubation at 70 MPa (8°C) colony growth was examined and found to be highly reproducible. However, when the same methodology was used for *P. profundum* SS9, except the pouches were incubated at 28 MPa (15°C), the optimum growth conditions for *P. profundum* SS9 in liquid culture, we found inconsistencies in colony growth (data not shown). To try to improve the reproducibility of the colony growth of *P. profundum* SS9 at high pressure, we tested different types of low temperature-gelling agar and agarose. The agar used in the original study had a gelling temperature of ~30°C [29] and we thought that this could have a damaging effect on *P. profundum* SS9 colony growth. However, when we tried different lower gelling temperature agars and agaroses, these appeared to liquefy after incubation in the pressure vessel at 28 MPa (15°C) (data not shown). Therefore, the original low temperature-gelling agar was used [29] but instead of replica plating colonies, we spotted defined aliquots of a defined cell density of *P. profundum* SS9 onto solid agar in square plates (Fig. 2). Using this methodology, we did observe a decrease in the colony forming ability of *P. profundum* SS9 at 0.1 MPa (15°C) (Fig. 2A) compared to the non-overlay control plates (data not shown) incubated under the same conditions. However, *P. profundum* SS9 shows substantially improved growth on the overlay plates after incubation at 28 MPa (15°C)

relative to atmospheric pressure (15°C) (Fig. 2B & A, respectively). Therefore, despite the detrimental effect of the overlay on colony growth, these findings show that this method can be successfully used to assess quantitative colony growth of *P. profundum* SS9 on solid agar. These results also show that *P. profundum* SS9 shows piezophilic growth on solid agar.

3. Conclusions

The genetic studies to date provide evidence that processes affecting both the DNA and bacterial cell envelope play critical roles in the piezophilic growth of *P. profundum* SS9 in liquid culture. Although, the precise functions of the genes disrupted in the *P. profundum* SS9 transposon insertion mutants, will need to be investigated in future studies the initial findings, combined with the other genetic studies, support a model whereby processes affecting the fidelity (RecD), replication (DiaA, RctB & SeqA) and structure of the DNA (H-NS) and the integrity of the outer membrane (RpoE-like locus, RseB) and membrane fluidity (FabF & monounsaturated fatty acids) are central to defining the piezophilic capacity of *P. profundum* SS9. Future studies, using the methodology for quantitative colony growth of *P. profundum* SS9 on solid agar at high pressure could also provide insights into potentially novel mechanisms involved in piezophilic-surface attached growth.

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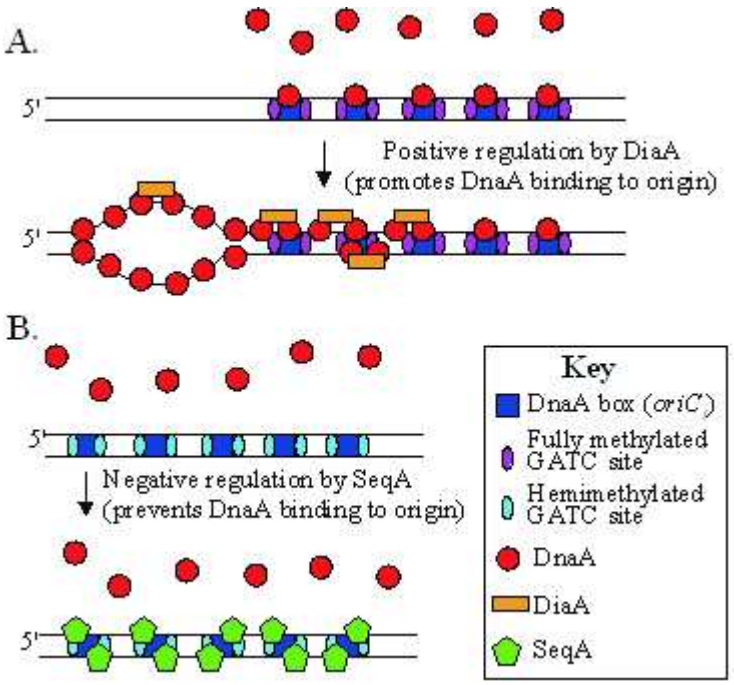
Legends

Fig. 1. Model outlining the proposed functions of putative DiaA & SeqA proteins in the initiation of replication of chromosome 1 in *P. profundum* SS9 & in piezophilic growth. (A) DiaA is a positive regulator of the initiation of chromosome 1 replication & removal of DiaA results in piezo-sensitive growth. (B) SeqA is a negative regulator of the initiation of the initiation of chromosome I replication & removal of SeqA results in piezo-enhanced growth. Model based on the functions of the *E. coli* DnaA, DiaA & SeqA proteins [17, 18, 20, 30].

Fig. 2. High pressure colony growth of *P. profundum* SS9. Cultures were grown to early stationary phase in marine broth, diluted to an OD_{600} ~0.8, then serially diluted and 10 µl aliquots spotted on 2.5% (w/v) low temperature-gelling marine agar (supplemented with 100 mM HEPES and 20 mM glucose). The square plates were incubated at 15°C (0.1MPa) for 2 hours, then overlaid with the marine agar as before. After cooling on ice, the agar sandwich was removed and heat-sealed in a sterilized plastic pouch. The pouches were incubated at 15°C at the defined pressures for 96 hours. For high pressure growth, the pouches were placed in a water-filled, temperature-controlled pressure vessel.



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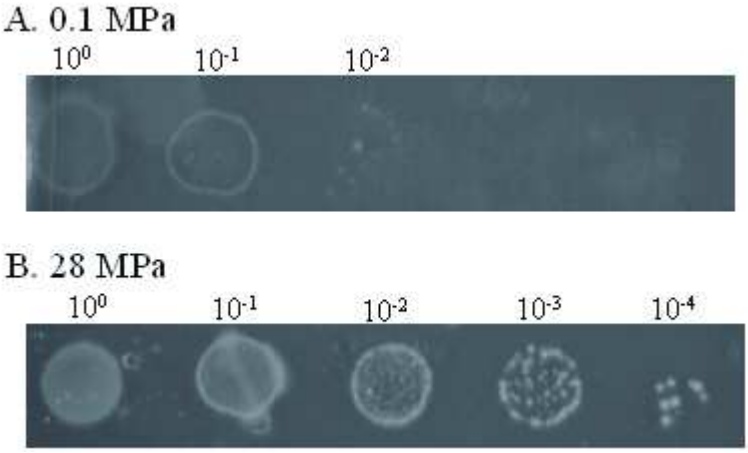

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